Cell death in prion disease

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Abstract

Prion diseases are a group of fatal neurodegenerative diseases, including CJD and scrapie, which are thought to be caused by a protein termed a prion (PrP). As manganese has previously been suggested to be involved in prion disease we have investigated manganese binding to PrP and its role in the toxicity of the protein. We have shown that manganese bound PrP (MnPrP) has several of the characteristics of the disease form of PrP, including protease resistance and toxicity that is dependent on cellular PrP expression. Further investigation into the mechanism of toxicity revealed that MnPrP is significantly more toxic to neuronal cells than non-manganese bound PrP and that toxicity requires the presence of known metal binding residues within the protein. We have demonstrated that treatment of neuronal cells with MnPrP causes caspase 3 activation and apoptosis, as demonstrated by DNA laddering, and we hypothesise that caspase 3 is activated by a p38 pathway. Treatment of neurones with MnPrP also caused a significant increase in cellular ROS production, although this did not appear to be a major cause of cell death as antioxidants were unable to save cells from cell death. We also investigated mechanisms by which cells can survive scrapie infection and MnPrP toxicity. We have shown that cells infected with scrapie have increased ERK activation which was important for their survival. Cells that survived MnPrP treatment were also found to have increased ERK activation. This suggests that ERK may have a protective role in prion diseases and may be a potential therapeutic target.

Abbreviations

AB: Amyloid beta

AD: Alzheimer's disease

ApoPrP: Water refolded PrP

ATRA: All-trans retinoic acid

BBB: Blood brain barrier

BDNF: Brain-derived neurotrophic factor

BSE: bovine spongiform encephalopathy (BSE),

CD: Circular dichroism

CGN: Cerebellar granule neuron

vCJD: Variant Creutzfeld Jacob Disease

sCJD: Sporadic Creutzfeld Jacob Disease

CMV: Cytomegalovirus

CNS: Central nervous system

CSF: Cerebrospinal fluid

DBPS: Dulbeccos phosphate buffered saline

DMEM: Dulbeccos Modified Eagles Media

DMSO: Dimethyl sulfoxide

EPR: Electron paramagnetic resonance

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinases

FBS: Foetal bovine serum

FFI: Fatal familial insomnia

GAG: Glycosaminoglycan

GFP: Green fluorescent protein

GPI: glycosylphosphatidylinositol

GSS: Gerstmann-Sträussler-Scheinker

HaPrP: Hamster PrP

HO-1: Heme oxygenase

HuPrP: Human PrP

IMAC: Immobilized metal ion affinity chromatography

IP(3)R: Inositol 1,4,5-trisphosphate

JNK: C-Jun N-terminal kinases

MAPK: Mitogen-activated protein kinases

MnPrP: Manganese bound PrP

MRE: Metal response element

MTS: Tetrazolium Salt

NCS: Newborn calf serum

NGF: Neuronal growth factor

NMR: Nuclear magnetic resonance

NO: Nitric oxide

PI3K: Phosphoinositide 3-kinases

PK: Proteinase K

PKA: Protein kinase A

PKC: Protein kinase C

PMCA: Protein-misfolding cyclic amplification

PMS: Phenazine methosulfate

PPS: Pentosan polysulphate

PrP*: Hypothesised PrP intermediary

PrP106: Mini prion

PrP 106-126: Widely used PrP peptide spanning amino acid 106-126 of HuPrP

PrP: Prion Protein

PrP^{c}: Cellular PrP (normal form)

PrP^{res}: Partially protease resistant PrP

PrP^{sc}: Abnormal PrP

RIPA: RadioImmunoPrecipitation Assay

RML: Rocky Mountain Laboratory

ROS: Reactive oxygen species

RyR: Ryanodine

SDS: Sodium dodecyl sulfate

SOD: Superoxide dismautase

TSE: Transmissible spongiform encephalopathy

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1. Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of diseases that affect mammals. The diseases lead to pathology within the central nervous system (CNS), characterised by the formation of protein deposits and the spongy appearance of the CNS tissue. This causes a wide variety of symptoms, including a lack of coordination leading to paralysis. TSEs are invariably fatal.

TSEs can occur spontaneously, through transmission and be inherited. One of the most recent transmissible TSEs to emerge is variant Creutzfeld Jacob Disease (vCJD). The first confirmed case of vCJD was reported in 1996 (Will et al., 1996). vCJD has an earlier onset than CJD and a longer illness duration (Will and Ward, 2004). It has been linked to bovine spongiform encephalopathy (BSE), another new TSE affecting cattle, through strain typing and it has been widely postulated that vCJD has been transmitted to the population through the consumption of cattle infected with BSE (Bruce et al., 1997). However no direct link between BSE and vCJD has been demonstrated. As well as the possibility that vCJD may be transmitted from BSE infected cattle, several cases of human to human transmissions have been reported (Hewitt et al., 2006). The fear that there may be a large number of people with subclinical vCJD has led to intensive research into TSEs.

There are also several inheritable prion diseases, due to mutations in the prion protein. These include Gerstmann-Sträussler-Scheinker (GSS) disease and fatal familial insomnia (FFI). GSS is caused by mutations of the *prnp* gene and follows a pattern of autosomal dominant inheritance. As with other TSEs, it is classified by the presence of amyloid plaques leading to neurodegeneration. FFI is also the result of mutations within the *prnp* gene and shows an autosomal dominant inheritance pattern, though with incomplete penetrance. As the name suggests patients suffer sleep disturbance in the earlier stages of the disease, which get progressively worse throughout the disease (for review see (Collins et al., 2001)).

In humans 80% of TSEs are sporadic. The most common TSE is sCJD which has an incidence of approximately 1 in a million (1996). Unlike vCJD, sCJD has a late onset, and a shorter duration. There are several other TSEs that occur sporadically, including scrapie which is endemic in sheep in most European countries including the UK. This is, perhaps, the earliest reported TSE, with reports of scrapie dating back to the eighteenth century (Brown and Bradley, 1998). Whilst scrapie occurs sporadically, work has shown that certain sheep genotypes are much more susceptible to scrapie than others, this has led to national breeding programme in the UK which aims to increase the number of resistant sheep in the UK flock to prevent outbreaks.

TSEs are thought to be caused by a protein termed the prion protein. This protein was first discovered in 1982 by Prusiner and is still unique, as it is the only protein thought to be a transmissible disease causing protein. The prion protein is a 30-39kDa protein that is expressed in most tissues, however the highest expression levels are found in neural and lymphoid tissue. In prion disease the protein becomes misfolded. There is an increase in β- sheet content and a corresponding decrease in α- helices (Pan et al., 1993). This structural change may lead to a loss of function and, perhaps most importantly for disease, protease resistance and an ability to aggregate (Prusiner et al., 1983).

The misfolded form of PrP is thought to be responsible for neurodegeneration in the TSE's although the mechanism underlying nuerodegeneration is still unclear. Current research focuses on finding possible therapies for the TSE's as there is currently no known cure.

1.1 PrP Structure and Expression

1.1.1 Genetic regulation of PrP

The prion protein is encoded by the *Prnp* gene which has been well characterised in several species. The gene consists of three exons and two introns, with the third exon encompassing the entire open reading frame. The exceptions to this structure are in humans and hamsters where the second exon may not be spliced into the final mRNA sequence (Lee et al., 1998; Li and Bolton, 1997). Studies of the mice and

bovine gene have shown the importance of intron 1 in the modulation of gene expression as well as exon 1 which can act as an inhibitor of PrP expression (Haigh et al., 2007). Interestingly a TATA box has been found in intron 1 of both the mouse and bovine gene (Haigh et al., 2007), this is in contrast to the prion promoters studied so far, which lack a TATA box. However various binding sites have been found within the promoter that enhance and inhibit promoter activity. These include Sp-1 binding sites and AP-1 and AP-2 binding sites, which can inhibit and enhance promoter activity depending on which transcription factors bind to them (Saeki et al., 1996b). Heat shock elements have been found in the rat promoter (Shyu et al., 2002), and heat shock proteins have also been shown to up-regulate prion protein expression in humans cells (Shyu et al., 2000).

A variety of other factors have also been shown to regulate PrP expression either at the protein or gene level. The prion protein has been hypothesised to have a role in copper homeostasis in the brain (for review see (Brown, 2001)) so it is perhaps unsurprising that copper has been shown to regulate PrP promoter expression in PC12 cells, a neuronal cell line. Deletion of parts of the promoter showed that a region of the promoter containing a putative MRE was essential for the upregulation of promoter activity in response to copper (Varela-Nallar et al., 2006). Copper has also been shown to up-regulate PrP expression at the protein level (Brown et al., 1997b). However another study has shown that copper can down regulate PrP gene expression in GN11 cells, another neuronal cell line (Toni et al., 2005). Studies looking at other metals have shown that manganese and zinc are unable to regulate the prion promoter (Varela-Nallar et al., 2006), suggesting that the response to copper by the prion promoter is a specific specialised response.

As the loss of the prion protein has been connected with oxidative stress, several studies have investigated the effect of hypoxia and ischemic brain injury on PrP expression. A study has shown that PrP expression is up-regulated in mice after severe focal ischemic brain injury, and that this increase in expression occurs shortly after brain injury. They hypothesise that given the known neuroprotective roles of PrP^c up-regulation of PrP^c may be protective (Weise et al., 2006). This is supported by a study demonstrating that PrP null mice show greater injury after ischemic injury when compared to WT mice (McLennan et al., 2004). Another study used hyperbaric

oxygen treatment to induce oxidative stress in N18 cells, a mouse neuroblastoma cell line. Treatment was shown to up-regulate PrP expression at both mRNA and protein levels. It was also shown to upregulate HSP70 expression, the upregulation of both these protein was shown to be regulated via JNK activation (Shyu et al., 2004). Overall there is strong evidence for a role for PrP^c in the prevention of oxidative stress in the brain and expression of PrP^c may be modulated by oxidative stress.

As PrP expression is important for disease progression studies have investigated compounds that can regulate PrP expression. All-trans retinoic acid (ATRA) is a powerful antioxidant that is currently licensed for the treatment of skin conditions such as acne and it is also used in the treatment of acute promyelocytic leukemia, due to its ability to differentiate leukemic promyelocytics into mature granulocytes (Huang et al., 1988). Interestingly, ATRA has also been shown to down-regulate prion expression in granulocytes, independently of granulocyte maturation, this has led to the suggestion that it may be useful therapeutically (Rybner et al., 2002).

1.1.2 Primary Structure

PrP is relatively small protein varying in size between 30-39kDa depending on species, it is highly conserved in mammalian species. In mice the *prnp* gene is found on chromosome 2 and encodes for a 254 amino acid sequence (figure 1.1).

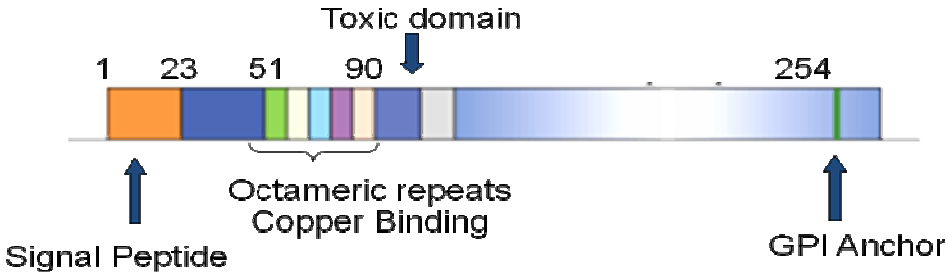


Figure 1.1: The major features of the primary structure of PrP.

A region spanning amino acids 1-23 directs the protein to the endoplasmic reticulum (ER) for processing and is cleaved shortly after translation. A short region at the end of the C-terminus is also cleaved during processing, prior to the addition of glycosylphosphatidylinositol (GPI) anchor. The GPI anchor tethers the protein to the

outside of the cell (Stahl et al., 1987). There are several important features in the primary structure of PrP. Perhaps most importantly for any possible function it has four octarepeats (PHGGGWGQ) in the N-terminal region of the protein, which have been shown to bind several transition metals including copper (Hornshaw et al., 1995) and manganese, although with a much lower affinity (Brazier et al., 2008; Brown et al., 2000). There is also a fifth metal binding site at amino acids 95 and 110 which binds both copper and manganese (Brazier et al., 2008; Thompsett et al., 2005). Amino acids 112-145 form a hydrophobic region that is thought to be involved in the pathogenesis of prion disease, as proteins missing residues 114-121 cannot be converted into PrP^{sc} when over-expressed in N2A cells infected with scrapie (Holscher et al., 1998). Parts of the hydrophobic region have also been shown to form B-sheets spontaneously as in the case of the 106-126 peptide (Forloni et al., 1993) or be amyloidogenic such as residues 112-119 (Jobling et al., 1999).

1.1.3 Secondary Structure

The structure several species of prion protein has been determined by NMR including mouse PrP (Lopez Garcia et al., 2000; Riek et al., 1996; Zahn et al., 2000). The C-terminus of PrP has been found to be highly structured (figure 1.2). The PrP^{c} form consists of three α- helices at amino acids 114-154, 170-193 and 200-217 with a disulphide bond linking helix 2 to helix 1. There are also two short strands of β-sheet structure at amino acids 128-131 and 161-193 (Riek et al., 1996). The structure of the N-terminus of PrP is much less clear, when it is not bound to metals. Studies using a variety of techniques including nuclear magnetic resonance (NMR) and circular dichroism (CD) have shown it is highly flexible (Hornemann et al., 1997), however it has been postulated that on binding to copper the N-terminus may become more structured (Jones et al., 2004; Miura et al., 1996). The sequence of mammalian prion protein is highly conserved especially the unstructured N-terminus indicating its importance in function (Wopfner et al., 1999).

Upon conversion to PrP^{sc} the secondary structure changes significantly. PrP^{c} has been shown to contain ~ 40% α-helical structure and ~ 3% β-sheet structure (Pan et al., 1993), whilst PrP^{sc} has been shown to contain ~ 30% α-helical structure and ~ 40% β-sheet structure (Pan et al., 1993) (figure 1.2). However, due to the tendency

of PrP^{sc} to aggregate, the actual structure of PrP^{sc} has not been determined, although recent developments have provided some details of the structure. Lu *et al* studied the structure of D178N huPrP90-231, which undergoes an autocatalytic conversion to the amyloid state, using hydrogen/deuterium exchange. They demonstrated the location of the β-sheet core was in the C-terminus of the protein (Lu et al., 2007). Higher resolution work using site-directed spin labelling and electron paramagnetic resonance (EPR) spectroscopy has shown the amyloid region to encompass residues ~160-220. The structure was found to be parallel in-register β–sheet (Cobb et al., 2007). This is in contrast with earlier modelling studies (DeMarco and Daggett, 2004; Govaerts et al., 2004) but the structure described is common motif for many amyloids including Aβ (Torok et al., 2002) and alpha synuclein (Chen et al., 2007).

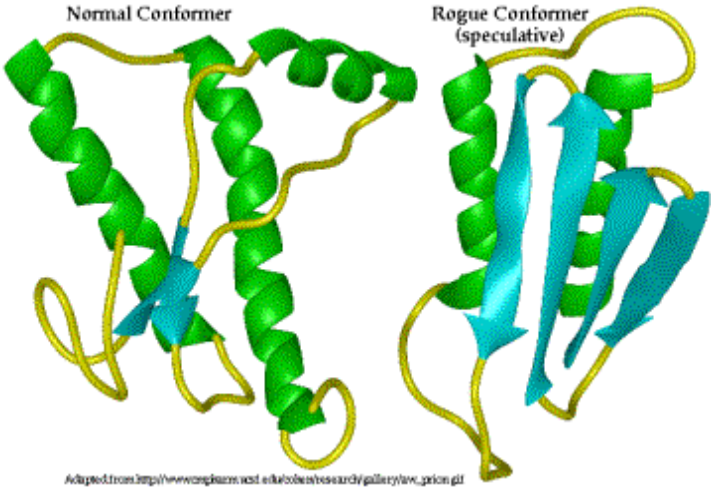


Figure 1.2: The secondary structural features of the prion protein. The normal conformer (PrP^{c}) of the prion protein is high in α-helical structure. The abnormal conformer (PrP^{sc}) has a much higher level of β-sheet content.

The prion protein sequence is highly conserved throughout mammalian species, but in non-mammalian species the sequence is much less well conserved. Prion homologues exist in many species, perhaps the best characterised of the non-mammalian prions are the prion proteins of chickens (Gallus Gallus), turtle (Trachemys scripta) and frog (Xenopus laevis). Using NMR the structure of these proteins has been identified, they show only about 30% homology to mammalian prion proteins but do retain the structured C-terminus and relatively unstructured N-terminus. The globular domain in chicken and turtle PrP consists of three α-helices and a short anti-parallel β-sheet. Xenopus PrP also contains three α-helices but has a

longer β-sheet region. Despite the low homology the structure of the globular C-terminus is remarkably similar to that of mammals suggesting that key amino acids are involved in structure (Calzolai et al., 2005).

Several regions of the N-terminus are also conserved between huPrP and the non-mammalian prion proteins, this includes amino acids 15–20 which are involved in the cleavage of the amino acids 1-22. However interestingly the octarepeat region is not highly conserved, in turtles and chickens the region consists of hexarepeats and in Xenopus no repeat region is present suggesting that this protein is not able to bind metal (Calzolai et al., 2005).

1.2 PrP conversion

The conversion of the prion proteins results in a dramatic change in both the properties of the protein and its structure. PrP^{sc} has a protease resistant core and is thought to be infectious.

Conversion of PrP can be explained as follows: PrP^{c} is thought to exist in equilibration with another intermediary termed PrP*, with PrP^{c} dominating the equilibrium (Cohen et al., 1994). PrP^{sc} is thought to bind to PrP* and convert it to PrP^{sc}, assembly of PrP^{sc} then continues until a stable seed is formed, the seed can continue to grow by accretion and can divide by breakage into smaller, infectious units. In transmitted disease this PrP^{sc} would come from exogenous sources (Figure 1.3). In inherited disease there may be a preference for the PrP* that can then be converted into PrP^{sc}. Once this process begins the amount of PrP^{sc} will increase exponentially (Cohen and Prusiner, 1998). Several studies have implicated another protein in this conversion process termed protein X (Kaneko et al., 1997; Telling et al., 1995).

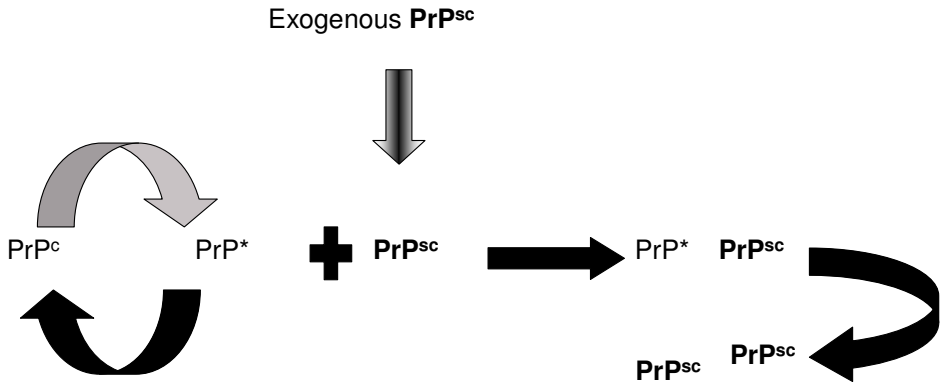


Figure 1.3: PrP conversion. PrP exists in two forms in PrP^{c} and PrP*. When exogenous PrP^{sc} binds to the much rarer form PrP* it is able to convert PrP* in to PrP^{sc}. This then begins an exponential increase in PrP^{sc}.

Protien X was first hypothesised to exist after experiments on transgenic mice expressing a chimeric form of PrP containing part of the mouse and human sequences. These mice were susceptible to human prions, unlike mice expressing both mouse and human prions which were resistant to human prions (Telling et al., 1994). This led to the theory that protein X bound to a specific part of PrP^{c} and assisted in its conversion. As mice expressing the mouse and human prions were resistant to human prion protein, protein X must bind more strongly to the mouse PrP. Further experiments elucidated the binding site of protein X, at the C-terminal end of the protein (Telling et al., 1995), however protein X has never been isolated.

1.3 PrP Function

Understanding the function of the prion protein may be essential in understanding its role in disease as the disease might result from a change in function of the prion protein, this could either be a gain of toxic function, a subversion of function for instance the ability to transduce pro-apoptotic signals rather than anti-apoptotic signals or a loss of function (for review see (Westergard et al., 2007)).

1.3.1 PrP in copper transport

Several studies have suggested that PrP may have a role in controlling synaptic copper fluctuations. PrP^c is predominantly located in the synapse (Herms et al., 1999) and is rapidly internalised in response to copper treatment *in vitro* (Brown and Harris, 2003; Pauly and Harris, 1998). PrP expression is also linked to copper release on depolarisation of the synapse (Brown, 1999). In PrP-null mice there is evidence of a reduction of copper in synaptosomal preparations (Brown et al., 1997a) and in the disease state there is evidence of a change in metal balance in the brains of mice infected with scrapie (Thackray et al., 2002), this may be due to the loss of the prion protein's ability to bind copper. The loss of the function of prion protein as a copper transporter protein, in the disease state, may result in copper imbalance in the brain. Both an increase or decrease of copper in the brain can lead to brain damage, this is demonstrated in two inheritable diseases, both due to mutations in copper transporters. Wilson's disease involves mutations in the ATP7A gene, ATP7A is a copper transporter and mutations result in an increase of copper in the brain and the liver, symptoms include liver disease and neurodegeneration. Menke's disease is also due to a mutation in the ATP7A gene and results in poor distribution of copper in the body, with low levels of copper in the brain, this also results in neurodegeneration (Ala et al., 2007; Menkes et al., 1962).

1.3.2 PrP as an antioxidant

PrP^c has been shown have superoxide dismutase (SOD) like activity. Studies have shown that recombinant PrP and PrP extracted from brain tissue has copper dependent SOD-like activity (Brown et al., 1999), this has led to the hypothesis that it may have an antioxidant role in the brain. However other studies have not been able to detect SOD like activity in recombinant protein (Jones et al., 2005b) and suggest any antioxidant activity may be due to indirect action.

Whilst the mechanism behind PrP^c antioxidant activity is unclear, it is clear that PrP^c plays a role in reducing oxidative stress in the brain. It has been shown that PrP knockouts have compromised antioxidant function (Klamt et al., 2001). Research has also shown that cultured neurones from mice deficient in PrP^c were more sensitive to

oxidative stress (Brown et al., 1997c). In the normal state PrP^c expression is increased in cells exposed to oxidative stress (Brown et al., 1997b).

In the disease state immunohistochemistry of brain slices of mice infected with scrapie have revealed several markers of oxidative stress including nitrotyrosine, a marker of peroxynitrite generation (Guentchev et al., 2000) and lipid oxidation markers (Wong et al., 2001c). In *vitro* experiments are in agreement with these results, oxidative stress was induced in cells treated with the 106-126 fragment of PrP (Pietri et al., 2006). The increase in oxidative stress markers in the disease state may be due to the conversion of PrP^c to PrP^{sc}.

1.3.3 Other Functions

As PrP^c is mainly found at the cell surface it has been suggested that PrP may have a role in signal transduction. In lymphocytes PrP^c has been shown to be incorporated into lipid rafts within the cell membrane, these rafts contain accumulated cross-linked proteins. It is hypothesised that PrP^c may become cross-linked in these rafts. PrP^c cross-linking has been shown to cause the activation of several signalling pathways including MAPK pathways (Stuermer et al., 2004). Several neuroprotective molecules have also been shown to be activated by a PrP^c binding partner including ERK and protein kinase A in mouse retinal cells, this activation was not seen in PrP null cells (Chiarini et al., 2002).

Some studies have suggested that PrP^c may have a role in regulating sleep patterns as patients suffering from FFI have disrupted sleep patterns. PrP null mice have also been shown to have altered sleep regulation when compared to WT mice (Tobler et al., 1997; Tobler et al., 1996). Recent work has shown that neuronal but not non-neuronal PrP^c expression is involved in sleep homeostasis and that PrP^c may play a role in the hormonal regulation of the hypothalamic–pituitary–adrenal axis (Sanchez-Alavez et al., 2007).

1.4 PrP and Metals

1.4.1 Copper

PrP has been shown to bind several transition metals with varying affinity. PrP has been shown to have the highest affinity for copper and has been shown to bind up to 6 copper atoms at physiological conditions. Copper is thought to bind at two sites, the octarepeat region and the so-called fifth site at 95 and 110. PrP has also been shown to bind several other metals including nickel, zinc and manganese. However the interactions between these metals are at a much lower affinity.

The prion protein has been demonstrated to bind copper at the octarepeat region both *in vitro* and *in vivo* (Brown et al., 1997a), although binding to the fifth site has only been demonstrated *in vitro* (Jones et al., 2004; Jones et al., 2005a). The affinity of copper for PrP is still widely debated with some studies suggesting affinity values as low as micromolar affinity (Stockel et al., 1998) and some as high as femtomolar affinity (Jackson et al., 2001). Most agree that there is at least one nanomolar-binding event (Treiber et al., 2007; Walter et al., 2006). In the octarepeat region copper the histidine is thought to be the primary residue coordinating binding, with the full complex consisting of residues HGGGW (Aronoff-Spencer et al., 2000; Burns et al., 2003), however the exact coordination is dependent on copper loading, with differing coordination patterns when less than two copper molecules are bound (Chattopadhyay et al., 2005).

The fifth site is now widely accepted to bind copper and there has been a significant amount of research into the coordination of this binding. Binding requires that at least amino acids 92-96 and 107-111 (in humans) are present (Jones et al., 2005a). They also showed that both pH and chain length effected the relative affinities of each histidine with the binding at low pH being coordinated whilst at higher pH the histidines bind independently (Klewpatinond and Viles, 2007). The affinity of copper for the 5^{th} site is also under debate however it has been shown that it has a higher affinity for copper than the octarepeat region (Jones et al., 2005a; Treiber et al., 2007).

Once converted to PrP^{sc} there is no evidence that copper can still bind to the protein however copper is still able to modulate prion disease. It has been shown that the copper chelator, D-(-)-penicillamine can delay the onset of prion disease in mice (Sigurdsson et al., 2003) and that brain homogenates incubated with $CuCl_2$ showed increased PK resistance. Conversely other studies have shown that treatment of scrapie infected cells with copper increases PrP^{c} expression but decreases PrP^{sc} accumulation, hamsters infected with scrapie also showed delayed onset of prion disease when treated with copper. This was hypothesised to be due to the increase in internalised PrP^{c}, which would hinder its interaction with PrP^{sc} (Hijazi et al., 2003).

Copper has been shown to be able to convert recombinant PrP into a PrP^{sc}-like protein (Qin et al., 2000), as well as being able to convert PrP^{c} from mice brains into a PK resistant form, although this was shown to be structurally distinct from PrP^{sc} (Quaglio et al., 2001). Further work has shown that copper is able to inhibit conversion of full length recombinant PrP by stabilising a PK resistant non-amyloidgenic form of PrP (Baskakov et al., 2002). However when copper was added to fibrils of PrP it increased PK resistance and induced further aggregation (Bocharova et al., 2005). The complexity of coppers involvement in the disease state in highlighted by the differing results obtained by groups and more research will need to be done to elucidate the role of copper and PrP both in health and disease.

1.4.2 Manganese

PrP has been shown to bind several transition metals as well as copper. Perhaps the most extensively researched of these is manganese. Early work suggested that the prion protein bound manganese in the octarepeat region of the prion protein and was able to bind up to 4 manganese atoms (Brown et al., 2000), however this was in contrast to other reports that suggested that manganese was unable to bind in the octarepeat region (Garnett and Viles, 2003; Treiber et al., 2007). Further work suggested that another binding site at histidines 95 and 110 (the so-called fifth site) was able to bind manganese however it was suggested that this site was a low affinity site when compared to the octarepeat region (Jackson et al., 2001). More recent work has used isothermal titration calorimetry to identify the manganese

binding sites in wild-type mouse PrP. This revealed that PrP has two manganese binding sites with affinities that would bind manganese at concentrations of 63 and 200 μM at pH 5.5, interestingly this affinity is similar to other known manganese binding proteins. In contrast to earlier work they concluded that the highest affinity manganese-binding site was associated with His-95 in the so-called "fifth site". They also established that manganese could bind at the fifth site in the presence of copper suggesting that copper displacement would not be required for manganese binding (Brazier et al., 2008).

The interest in manganese binding PrP is due to several lines of evidence that suggest that manganese may have a role in prion disease. Elevated manganese levels have been observed in the CNS and blood of the patients suffering from prion disease, suggesting that manganese may play role in disease (Hesketh et al., 2008). This phenomenon has also been observed in scrapie and BSE (Hesketh et al., 2007), and as the increase in manganese occurs prior to the onset of clinical signs of disease it has been suggested as a possible diagnostic tool (Hesketh et al., 2007; Thackray et al., 2002).

Levels of manganese have also been shown to be significantly higher in the brain tissue of patients with sCJD. The metal occupancy of PrP in sCJD patients also differed from PrP purified from normal brains. There was an elevation of manganese and, to a lesser extent, of zinc accompanied by significant reduction of copper bound to the purified PrP (Wong et al., 2001b). The reason for the change in metal balance in the brains of TSE sufferers remains unclear. It was hypothesised that high dietary manganese may result in changes in manganese concentration in the brain and therefore manganese binding to PrP. However research into this hypothesis has shown high dietary manganese does not result in increased susceptibility to prion disease (Legleiter et al., 2007a; Legleiter et al., 2007b). Work investigating a link between high levels of soil manganese and scrapie ‘hotspots’ has shown no correlation between the two (Chihota et al., 2004). It is likely that the reason for the metal imbalance observed in prion disease is more complex than a simple dietary imbalance.

Manganese binding has been shown to convert the prion protein into a structure more like that of PrP^{sc}. Investigation has shown that initially manganese binding resulted in a protein similar to copper bound PrP, however after ageing the protein became protease resistant and lost function. The protein also underwent structural changes resulting in an increased β-sheet content, manganese binding also resulted in increased toxicity to astrocytes (Brown et al., 2000). More recent work has shown that initial manganese binding to PrP results in an increase in α- helical content suggesting that ageing is required for conversion to a more PrP^{sc}-like protein (Zhu et al., 2008). It has also been shown that manganese binding to PrP does not protect manganese from water interactions, unlike copper binding, which protects copper from water interactions. A real-time study of the protein alloforms showed that copper bound PrP remained stable in solution, but that manganese bound PrP underwent different changes that led to fibril formation (Tsenkova et al., 2004).

In vitro work has shown that, in common with several other transition metals manganese influences PrP aggregation. Work in yeast has shown that manganese supplemented media induced PrP^{res} formation (Treiber et al., 2006). Manganese has also been shown to have pro-aggregatory effect on PrP in cell-free systems, which could be blocked by copper (Giese et al., 2004). Further work using the protein-misfolding cyclic amplification (PMCA) cycle, a technique that has been shown to drive the amplification of misfolded prion protein of PrP^{Sc} seeds, showed that treating normal hamster brain homogenate with manganese was able to efficiently drive PrP conversion. This suggests that manganese could be involved in conversion *in vivo* (Kim et al., 2005). In contrast other work using cell free systems has shown that manganese has no effect on PrP conversion or aggregation (Bocharova et al., 2005).

Work investigating the mechanism of aggregation of PrP in cells treated with manganese has suggested that it is due to manganese inducing reversible intermolecular binding between PrP fibrils rather than manganese binding to the protein (Levin et al., 2005) suggesting that aggregation induced by manganese treatment occurs by a different route that of manganese loaded PrP, which appears to occur because of structural changes.

Unlike copper, manganese is unable to induce cellular prion protein expression (Varela-Nallar et al., 2006), suggesting that PrP is not involved in manganese metabolism. However the prion protein has been shown to protect against manganese toxicity; PrP^{c} has been shown to reduce manganese accumulation in cells treated with manganese and to protect against manganese induced oxidative stress, as well as prevent manganese induced apoptosis (Choi et al., 2006). However work using a different cell line showed that PrP^{c} did not protect against manganese toxicity (Rachidi et al., 2003).

There is increasing evidence that manganese binding to the prion protein results in conversion of the prion protein to a PrP^{sc}-like protein. Recent work has demonstrated the potential relevance of this *in vivo*, as manganese levels are heightened in patients suffering from CJD. Work demonstrating that the binding affinity of PrP for manganese is similar to that of other known manganese binding proteins, demonstrates the physiological relevance of manganese binding to PrP.

1.4.3 Prion Protein and other Transition Metals

As well as manganese binding to the prion protein, iron binding has also been hypothesised to be involved in the disease, as studies have shown higher levels of iron in scrapie infected hamsters (Kim et al., 2000). There is also evidence that iron can covert PrP^{c} to PrP^{sc}- like form and that when infected cells are iron depleted there is a decrease in the production of PrP^{sc} (Basu et al., 2007).

Metals have also been shown to modulate aggregation of the prion protein and studies using PrP 106-126 have shown that both copper and zinc could bind to the peptide leading to aggregation and neurotoxicity. This effect was reversed when the metals were removed (Jobling et al., 2001). The evidence that metal imbalance is a feature of several neurodegenerative diseases and that both iron and manganese have been shown to convert PrP to a PrP^{sc}-like protein lead many to belief that metals may be involved in the pathogenesis of prion disease.

1.5 Prion Protein Toxicity

Prion disease results in massive neuronal cell death, leading to the classic spongiform appearance in the brain. However, despite intensive research, there is no clear answer to the mechanism underlying the toxicity of the prion protein. Neurotoxicity is unlikely to be wholly due to the loss of function of the prion protein as PrP null mice have few abnormalities (Bueler et al., 1992). Although as conversion from PrP^{c} to PrP^{sc} is thought to result in loss of function it may play some role in toxicity (Hetz et al., 2003a).

It is unclear whether PrP^{sc} itself is toxic although *in vitro* studies have demonstrated that PrP^{sc} is toxic (Hetz et al., 2003b). However some forms of prion disease have very low levels of PrP^{sc} deposits in the brain (Collinge et al., 1995a; Hsiao et al., 1990; Manetto et al., 1992) leading to suggestions that PrP^{sc} is not toxic itself but simulates neurotoxic signalling pathways (Solforosi et al., 2004), or that only a subset of PrP, generated as an intermediate (PrP*) or as a side product during prion propagation, is toxic (Hill et al., 2000; Manuelidis et al., 1997).

1.5.1 PrP Expression and Toxicity

It is clear that toxicity requires cellular PrP expression as PrP-null neurons survive exposure to a fragment of the prion protein (Brown et al., 1994). Cellular prion expression has also been shown to be required *in vivo* for toxicity. PrP null mice that received neural grafts over-expressing PrP^{c} were inoculated with mouse adapted scrapie and whilst the grafts became infected and developed the severe histopathological changes characteristic of scrapie the PrP-null tissue remained healthy (Brandner et al., 1996). The level of PrP^{c} in the cell has also been shown to effect the incubation time of the disease with lower expression levels leading to a prolonged incubation time (Manson et al., 1994; Prusiner et al., 1993). PrP^{c} is up-regulated in disease (Voigtlander et al., 2001) suggesting that PrP^{sc} itself may have and up-regulatory effect on the promoter region of *prnp*. Therefore gene expression, in particular the activity of the *prnp* promoter are thought to be important in disease pathogenesis.

1.5.2 Regions Required for Infection and Toxicity

Investigation has revealed the requirement for certain regions of cellular prion protein for prion propagation. The hydrophobic region amino acids 112-119 is the most fibrillogenic region of the prion protein and has been shown to be required for prion infection *in vitro* (Norstrom and Mastrianni, 2005). Studies have also shown that PrP^{Sc} interacts with this region suggesting its importance in conversion (Brown, 2000b).

Numerous studies have also shown that amino acids 23-90 are not required for prion propagation (Fischer et al., 1996; Muramoto et al., 1997; Muramoto et al., 1996) suggesting that much of the N-terminal region is not required for infectivity. Further work using a fragment of PrP termed PrP106 or the mini prion, which is 106 amino acids long and contains the deletions Δ23-88 and Δ141-176, has shown that the octareapet region, which spans amino acids 51-90, is not required for toxicity or infection (Bonetto et al., 2002). As the octarepeat region is the major metal binding region, and hence thought to be important for normal function, it is perhaps surprising this region is not required for infectivity.

The ablation of certain regions of the prion protein in transgenic mice are lethal which suggests that these regions may be involved in the toxicity of the protein, although the exact reason for the toxicity of the deletions remains unclear. Deletion of the N-terminus of the prion protein (amino acids 32-134) in transgenic mice results in a fatal pathology including ataxia, degeneration of cerebellar granule cells, and vacuolation of white matter in the brain. These symptoms are thought to be due to the up-regulation of Bax dependant pathways (Li et al., 2007a). Deletion of the region spanning 105-125, which encompasses the hydrophobic region, results in fatal neurodegeration in mice (Li et al., 2007b). Unlike N-terminal deletion, this deletion does not up-regulate Bax dependant pathways (Li et al., 2007a) but they hypothesise a model in which this protein has greatly enhanced affinity for a hypothetical receptor that serves to transduce the toxic signal (Li et al., 2007b). The deletion of amino acids 94-134 of the prion protein results myelin degeneration which led to the hypothesis that the prion protein is involved in maintaining myelin integrity (Baumann et al., 2007).

In summary the C-terminus of the prion protein appears to be important for infectivity, the hydrophobic region has been shown to be required for infectivity, as have amino acids 89-140 and 176-231. Deletion of regions of the prion protein, in transgenic mice, has shown that deletion of certain regions can be lethal, suggesting these regions may be important structurally.

1.5.3 Species Barrier and Conversion

A strong species barrier exists between most species, in most cases transmission from one species to another results in longer incubation time or is unsuccessful, however subsequent transmission resemble 'within species' transmission. Work using transgenic mice expressing hamster PrP^{c} showed that they are highly susceptible to Sc237 hamster prions, unlike WT mice (Prusiner et al., 1990). Studies argued that the barrier was due to in differences in PrP primary structure between the donor and recipient species, making it difficult for the conversion to occur (Palmer et al., 1991; Prusiner et al., 1990). However the BSE prion strain is highly promiscuous and retains its biological characteristics on transmission to other species (Bruce et al., 1994). Also, unlike classical CJD, vCJD transmits easily to mice (Hill et al., 1997), and as the CJD and vCJD variants have exactly the same structure it suggests be that the barrier is not species dependant. Recent work has also shown that, outside of the cell, mouse and hamster full-length recombinant PrPs are able to cross-seed polymerisation reactions furthering the suggestion that factors other than the PrP sequence may have an impact in regulating prion transmission between mammalian species (Makarava et al., 2007). However other studies have shown the maintenance of a species barrier in a cell-free environment, although this work used a fragment of PrP. This may suggest that this fragment contains the regions important in species barrier (Jones and Surewicz, 2005; Vanik et al., 2004).

1.5.4 Disease Mutations and Toxicity

Inheritable prion diseases result from a mutation in the prion protein genetic sequence that presumably makes the protein more likely to convert to PrP^{Sc}. The N-terminus of the prion protein region includes mutations involved in inheritable prion diseases, suggesting that this region may play an important role in the conversion of the protein to the neurotoxic form although previous work has shown that much of

this region is not required for conversion to PrP^{sc} (Muramoto et al., 1996). Perhaps the best described mutation is found in GSS patients at amino acid 102 where a proline is substituted for a leucine (Hsiao et al., 1989). The mutation has been reproduced in mice and upon challenge with scrapie results in prion disease (Hsiao et al., 1991). However high expression levels of PrP are required for disease to develop (Hsiao et al., 1991). In familial CJD, additional N-terminal mutations are observed which include multiple repeats of the octarepeat region. This highlights the importance of the metal binding regions in disease, even though this region is not generally thought to be required for infection (Flechsig et al., 2000). A mouse model containing insertions of extra octarepeats has been created. This model, Tg(PG14), contains an extra 9 repeats which corresponds to the largest number of extra repeats so far found in patients (Chiesa et al., 1998). These mice spontaneously develop prion disease.

In addition, several mutations involved in inheritable disease are found in the C-terminus of the prion protein, including E200K and F198S. It has been revealed that expression of certain parts of the C-terminus of PrP is intrinsically toxic to cells, and that this toxicity can be enhanced by mutations of the C-terminus of PrP that are found in genetic prion disease (Daniels et al., 2001), and that this toxicity is hypothesised to be due to the generation of hydrogen peroxide (Turnbull et al., 2003b). As with the C-terminal mutations described some other mutations have been directly linked to toxicity. Substitution of alanine for valine at amino acid 117 is a mutation observed in GSS and has been shown to increase the toxicity of PrP 106-126 (Brown, 2000a).

1.5.5 Recombinant Protein and Peptide Models of Toxicity

Perhaps the best-studied model of prion toxicity is the 106-126 peptide, which encompasses the hydrophobic region of the protein (amino acids 112-119). The 106-126 peptide has been shown to reproduce many of the properties of the prion protein, it is toxic to neuronal cells (Forloni et al., 1993), toxicity is mediated by cellular PrP expression (Brown et al., 1994) and requires the presence of mircoglial cells (Brown et al., 1996). Toxicity requires the presence of metals (Jobling et al., 2001) and the presence of copper results in the production of hydrogen peroxide, which may

explain the mechanism of toxicity of PrP 106-126 (Turnbull et al., 2003a). Furthermore, the 106-126 peptide induces neuronal apoptosis (Forloni et al., 1996), which is a feature of prion diseases *in vivo*. It is thought the hydrophobic core of the peptide mediates the toxicity and structure of the peptide, as substitution of the hydrophobic residues results in a decrease in toxicity as well as a decrease in β-sheet structure and alters the aggregation properties of the peptide (Jobling et al., 1999).

Other peptides that have been used as a substitute for PrP include a peptide spanning amino acids 185-208 which has a structural motif homologous to a motif in Alzheimer's disease-associated peptide Aβ (Klajnert et al., 2006). This fragment was shown to form amyloid fibrils in the presence of heparin and was also found to be cytotoxic (Cortijo-Arellano et al., 2008). A synthetic peptide spanning the sequence 82-146, which spans a fragment of the protein commonly found in patients suffering from GSS, has also been shown to readily form aggregates that are partially resistant to protease digestion (Salmona et al., 2003). This peptide has also been shown to be toxic to primary neurones and this is primarily due to the oligomers that it forms (Fioriti et al., 2007).

Longer fragments of the prion protein such as the mini prion have also been widely used to investigate prion toxicity. The mini prion is a soluble form of PrP that possesses several properties in common with PrP^{sc} (Bonetto et al., 2002; Laws et al., 2001). When expressed in scrapie infected cells, the mini prion can generate a protease-resistant polypeptide (Muramoto et al., 1996; Supattapone et al., 1999). *In vivo* PrP knockout mice expressing PrP106 are able to maintain scrapie infection (Supattapone et al., 1999). Work using recombinant mini prion has show that it is was highly toxic to primary neuronal cultures (Bonetto et al., 2002), thereby highlighting its usefulness as a model to study toxicity.

Many studies use the 90-231 fragment of PrP, this region is equivalent to PrP 27-30, the N-terminally truncated form of PrP. Both PrP 27-30 and recombinant 90-231 PrP were found to be toxic *in vitro* (Post et al., 2000). More unusually transmembrane or cytosolic prion protein has been used to investigate prion protein toxicity. A peptide

spanning 118-135 of PrP has been used to mimic prion toxicity as it is part of the putative transmembrane region of PrP. This region which has been shown to be toxic to neurones both *in vitro* and *in vivo* although toxicity is independent of PrP expression, which suggests this may not be relevant *in vivo* (Chabry et al., 2003). Additionally, although cytosolic PrP has been shown to be toxic *in vivo* this toxicity is also independent of cellular PrP expression (Norstrom et al., 2007) and other reports have found it is not toxic (Fioriti et al., 2005). As the toxicity of transmembrane and cytosolic PrP is independent of cellular PrP expression the value of these, as models of prion disease, is questionable.

Previous studies using recombinant prion protein have often used protein that has been converted into agregatory mis-folded and protease resistant forms like PrP^{sc} using metals (Kim et al., 2005) or SDS (Stohr et al., 2008). However SDS would not be found in tissue and as such does not represent a conversion that could take place *in vivo*. However studies using recombinant PrP have shown it to be a useful analogue of PrP^{sc}. PMCA cycling is common method of creating large quantities of PrP^{res}, a protease resistant form of PrP analogous to PrP^{sc}. PMCA cycling uses large quantities of PrP^{c} which are incubated with much lower concentrations of PrP^{res} which acts as template for the conversion of the PrP^{c} to PrP^{res}. The protein is sonicated at intervals to generate multiple smaller units for the continued formation of new PrP^{res} (Saborio et al., 2001). Protein generated in this manner has been shown to be selectively toxic to cells expressing PrP (Novitskaya et al., 2006) suggesting that recombinant PrP represents a realistic model of prion toxicity, whilst peptides often do not replicate a fragment of the peptide that would be found in the disease state.

Studies using recombinant prion protein have highlighted the link between aggregation, structure and toxicity. Emerging theories are centralised around the idea that prion toxicity is linked to prion structure, and the conversion of the prion protein to PrP^{sc} results in an increased tendency to form aggregates. There is increasing evidence that larger aggregates of PrP^{sc} are not toxic, as originally believed, but that the smaller soluble oligomers are the toxic species (Kazlauskaite et al., 2005; Simoneau et al., 2007). Small aggregates of other non-disease associated protein

have also been shown to be toxic suggesting that there may be a general mechanism of toxicity and avoidance of protein aggregation is crucial for the preservation of biological function (Bucciantini et al., 2002). Investigations using an antibody developed specifically against Aβ oligomers found the antibody was able and prevent their toxicity in vitro which demonstrates there is a common structure for soluble amyloid oligomers and also implies there is a common toxic mechanism (Kayed et al., 2003). This mechanism has since been suggested to involve increased membrane permeability and calcium dysregulation (Demuro et al., 2005). However, some argue that whilst oligomers are toxic to cells, they are not the sole toxic species, as mature fibrils have also been demonstrated to be toxic to cultured and primary cells (Novitskaya et al., 2006; Novitskaya et al., 2007).

There is increasing evidence that structure plays an important role in toxicity and studies have shown that amyloid proteins have similar structural features (Kayed et al., 2003). The toxicity of 90-231 is dependent on structure: α-helical 90-231 PrP is not toxic to cells but once converted to a β-sheet form the protein becomes more prone to aggregation and is toxic to cells (Corsaro et al., 2006). Further study has shown that denatured monomeric PrP 90-231 is toxic to cells, and that this is probably due to exposure of the hydrophobic region and increased internalisation (Chiovitti et al., 2007). The toxicity of full-length recombinant PrP also requires high β-sheet content (Novitskaya et al., 2006).

1.6 Cell death in Prion disease.

All TSEs result in a spongiform appearance in the brain due to the loss of neurones. Neuronal cell death in prion disease is widely accepted to be caused by apoptosis. This has been demonstrated both *in vitro* and *in vivo* in a variety of animals and in humans (Fairbairn et al., 1994; Forloni et al., 1993; Giese et al., 1995; Gray et al., 1999). The cause of apoptosis is still unknown, it may be due to a variety factors including a loss of function of PrP, aggregation of PrP or endoplasmic reticulum stress due to a build up of misfolded protein.

It is unclear how apoptosis in prion disease is initiated; PrP^{sc} may bind to receptors to initiate apoptosis as PrP has been shown to bind several receptors. Perhaps the most widely studied is the laminin receptor which has a role in PrP^{c} internalisation

(Gauczynski et al., 2001) and may have a role in PrP^{sc} propagation (Leucht et al., 2003). However no receptor has been shown to bind PrP^{sc} and initiate apoptosis. PrP^{c} may itself be involved in signalling, it has been shown to bind Fyn, a tyrosine kinase (Mouillet-Richard et al., 2000), PrP^{sc} may cause deregulation of this signalling leading to apoptosis (Pietri et al., 2006). However apoptosis may also be initiated internally by the endoplasmic reticulum and the mitochondria.

Studies have detected active caspase 12 in N2a cells treated with purified scrapie from mouse brain (Hetz et al., 2003b). As caspase 12 is localised to the endoplasmic reticulum (ER) and is associated with apoptosis induced by ER stress it suggests that the ER may play a role in apoptosis in prion disease. However more recent work has shown that ER stress may not have a prominent role in human prion diseases *in vivo* (Unterberger et al., 2006). Further research has shown that BiP a chaperone known to bind PrP is up-regulated in early prion disease, inhibition of BiP in N2A led to increased PrP^{sc} toxicity (Hetz et al., 2005) suggesting BiP may have a protective role. Other studies have shown that neurons treated with the 106-126 peptide release calcium through ER ryanodine (RyR) and inositol 1,4,5-trisphosphate (IP(3)R) receptors leading to ER stress and subsequent apoptosis via mitochondrial cytochrome C release (Ferreiro et al., 2008a).

p53 is a transcription factor involved in the regulation of cell cycle. PrP^{c} has been shown to regulate p53 induced caspase 3 apoptosis, in neuronal cells, leading to knock-out cells showing increased resistance to staurosporine induced apoptosis (Paitel et al., 2004). This has been shown to be regulated by C-terminal fragment of PrP (Sunyach et al., 2007). In the disease state, p53 has been shown to induce caspase 3 mediated cell death. Inhibition of p53 reduced caspase 3 expression, in hamster brains but had no effect on the incubation time of the disease (Engelstein et al., 2005), suggesting that this pathway is not required for cell death in prion disease.

The Bcl-2 family proteins comprise both anti-apoptotic and pro-apoptotic signalling molecules; they regulate apoptosis via the release of factors such as cytochrome-C from the mitochondria. Bcl-2 an anti-apoptotic signalling molecule and Bax a pro-apoptotic signalling molecule have both been implicated in prion toxicity. Over-expression of Bcl-2 in GT1 cells protects against the neurotoxic effects of the 106-

126 peptide (Ferreiro et al., 2007). *In vivo* Bcl-2 over-expression has been shown to protect mice expressing a lethal N-terminal truncated form of PrP (PrPΔ32-134), leading to an increase in life expectancy (Nicolas et al., 2007). Other studies have investigated the role of Bax, a pro-apoptotic member of the Bcl-2 family in prion disease. In the normal state there is evidence that PrP^c can protect against Bax induced apoptosis in a variety of cell including breast carcinoma and primary neuronal cells (Roucou et al., 2005). Bax deletion delayed cell loss in one mouse cell line expressing the lethal N-terminally truncated PrPΔ32-134 in contrast Bax deletion did not affect cell loss in another mouse cell line expressing the deletion mutant PrPΔ105-125 . Work using mice infected with the Rocky Mountain Laboratory (RML) strain of scrapie has also shown that deletion of Bax has little effect on disease progression and that over-expression of Bcl-2 also had little effect of disease progression (Li et al., 2007a). This suggests that the Bcl-2 family is not an appropriate target for therapy.

Several other pathways have been implicated in neurodegeneration in prion disease. pJNK is up-regulated in cells treated with the toxic peptide, 106-126 (Carimalo et al., 2005), *in vivo* pJNK is up-regulated in the brains of hamsters infected with scrapie. pERK (phosphorylated ERK) is also up-regulated in hamsters infected with scrapie (Lee et al., 2005). p38 activation has also been demonstrated in several models (Corsaro et al., 2006; Corsaro et al., 2003; Lee et al., 2005; Thellung et al., 2002). The activation of p38 may lead to caspase 3 activation and apoptosis as inhibition of p38 has been shown to prevent cell death in cells treated with PrP106-126 (Corsaro et al., 2003).

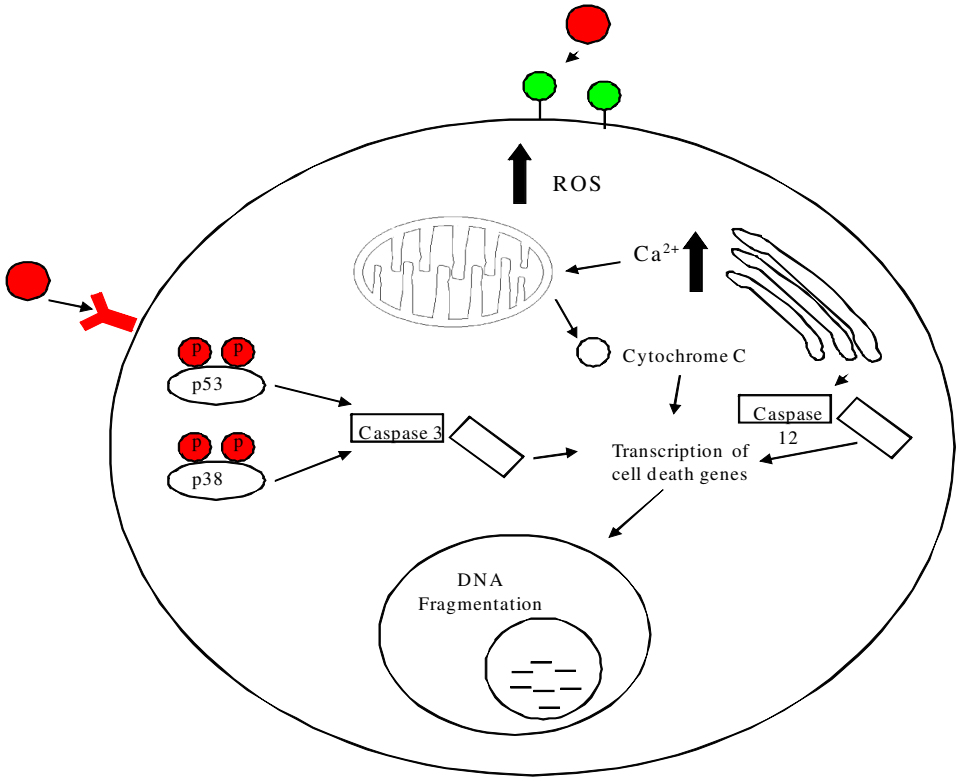


Figure 1.4: Cell signalling pathways thought to be involved in apoptosis in prion disease. The red circle represents PrP^{sc} and the green circles PrP^{c}.

1.7 Aims and Objectives

Despite extensive research into the toxicity of the protein the mechanism underlying the toxicity and conversion of the prion protein still remain a mystery. We have investigated the use of manganese bound PrP as model of prion toxicity. This model has allowed us to investigate the toxicity of the prion protein and gain a better understanding of the mechanism of prion toxicity. Initially we characterised manganese bound PrP and confirmed it replicated several properties of the prion protein, including protease resistance.

We investigated the regions of the prion protein required for MnPrP (manganese bound PrP) toxicity to try and further understand the effect of manganese binding on the prion protein. We established that metal binding to known metal binding residues was required for MnPrP toxicity. We also established that the octarepeat region had a protective effect on MnPrP and its removal resulted in increased toxicity.

We also wanted to establish the signaling mechanisms involved in cell death in MnPrP treated cells as well as using chronically infected cells to investigate pathway that may be involved in neuronal survival in scrapie infection, we hoped this might provide better understanding and possible therapies. We found that MnPrP caused apoptosis and the cell survival signal ERK was involved in cell survival in cells chronically infected with scrapie.

The final part of the study has investigated possible mechanisms for the prevention of manganese bound PrP toxicity, investigating the whether the promoter is up-regulated by MnPrP and whether this results in toxicity. We have also investigated whether antioxidant can protect against prion disease. Finally we have investigated whether the expression of non-mammalian prion proteins was protective against manganese PrP toxicity.

2. Materials and methods

2.1 Protein Production

2.1.1 Materials

LB Broth high salt purchased from Melford (Ipswich UK)

Qiagen Miniprep Kit from Qiagen (Crawley, UK)

Chelating Sepherose, Fast Flow from Amersham Biosciences (Buckinghamshire, UK)

Bio-Rad protein assay from Bio-Rad (Herts, UK)

Bovine serum albumin purchased from Fisher Scientific (Loughborough, UK)

Manganese Sulphate purchased from Fisher Scientific (Loughborough, UK)

Imidazole purchased from Fisher Scientific (Loughborough, UK)

Vivaspin concentrator purchased from Fisher Scientific (Loughborough, UK)

Triton X-100 purchased from Sigma-Aldrich (Poole, UK)

Igepal CA-630 purchased from Sigma-Aldrich (Poole, UK)

Nickel Sulphate purchased from Fisher Scientific (Loughborough, UK)

Copper Sulphate purchased from Fisher Scientific (Loughborough, UK)

Tris-HCl purchased from Sigma-Aldrich (Poole, UK)

EDTA purchased from Sigma-Aldrich (Poole, UK)

NaCl purchased from Fisher Scientific (Loughborough, UK)

PMSF purchased from Sigma-Aldrich (Poole, UK)

Lysozyme purchased from Sigma-Aldrich (Poole, UK)

Urea purchased from Melford (Ipswich UK)

Deoxycholic acid purchased from Sigma-Aldrich (Poole, UK)

DNase purchased from Sigma-Aldrich (Poole, UK)

BL21 cells purchased from Invitrogen (Paisley, UK)

XL-2 Blue ultra competent cells purchased from Stratagene (Amsterdam, Netherlands)

Carbenicillin purchased from Melford (Ipswich UK)

Kanamycin sulphate purchased from Melford (Ipswich UK)

IPTG purchased from Apollo Scientific (Stockport, UK)

Agarose purchased from Melford (Ipswich UK)

TEMED purchased from Sigma-Aldrich (Poole, UK)
SDS purchased from Fisher Scientific (Loughborough, UK)
Acrylamide purchased from National Diagnostics (Hessle, UK)
Dialysis Tubing purchased from Visking (UK)
Ethanol purchased from Fisher Scientific (Loughborough, UK)

2.1.2 Protein expression

2.1.2.1 Minipreps

Transformed XL-2 Blue ultra competent cells from a glycerol stock were spread onto an agar plate containing the appropriate selection antibiotic (either carbenicillin for pET23a plasmids or kanamycin for pEGFP-C1 and pd2-EGFP-N1 plasmids) and incubated overnight at 37^{o}C. A single colony was picked and incubated overnight in 3.5 ml of LB containing appropriate antibiotic, with shaking. The cells were spun down at 4000rpm for 10 minutes. Plasmid DNA was obtained using a Qiagen Miniprep Kit. The manufacturer's instructions were followed and the DNA was resuspended in 50µl of water. The product was run on a 1% agarose gel to check purity and determine quantity.

2.1.2.2 Protein Expression

Constructs had previously been created and inserted into a pET23a plasmid. DNA was transformed into BL21 cells as follows; cells were defrosted on ice then 1-2µg of plasmid DNA was added to the cells, the cells were then left on ice for 5 minutes. The cells were heated for 1 minute at 42^{o}C then placed on ice for 5 minutes. Cells were grown in 5mls LB for 1hr at 37^{o}C, 200 rpm. The culture was transferred into 20mls of LB broth and incubated overnight at 37^{o}C, 200rpm. The LB was supplemented with 50µg/ml carbenecillum. The next day the 20mls was added to a litre of LB supplemented with 50µg/ml carbenecillum and incubated at 37^{o}C, 200rpm until the cells reached and optical density of 0.6 (at 600nm). The cells were then supplemented with 1mM IPTG to induce protein expression. The cells were incubated for a further 4-5h and then spun down at 6000rpm for 15 minutes and stored at –20^{o}C prior to lysis.

2.1.3 Protein Purification

2.1.3.1 Cell Lysis

The transformed BL-21 cells were lysed to release protein as follows, the cells were resuspended in cell lysis buffer (50mM TrisHCl, 1mM EDTA, 100mM NaCl) with 0.4µM/ml PMSF, 10mg/ml lysozyme was then added to the lysis buffer and the cells were left at room temperature for 20 minutes. Deoxycholic acid was added at 2mg/ml and the cells were incubated at 37^{o}C, with shaking, until viscous. 20µg/ml DNase was then added to degrade any DNA present. The lysate was then spun at 12000rpm for 20 minutes. The supernantant was discarded and the pellet resuspended in lysis buffer, the lysate was then spun as before. The pellet was resuspended in binding buffer (8M urea, 200mM NaCl, 50mM Tris at pH 7.8) and stored at –20^{o}C.

2.1.3.2 Purification of His tagged Proteins

Protein was purified using immobilised metal ion affinity chromatography (IMAC) as previously described (Cui et al., 2003). A column was prepared as follows: it was packed with a bed of 6mls of chelating sepharose and washed with 5 volumes of water to remove the storage buffer. The column was then washed with 0.3M nickel sulphate which bound to the sepharose, it was then washed with MilliQ water to remove any excess nickel. It was then saturated with binding buffer prior to the addition of the lysate. The lysate was loaded onto the column and any his-tagged protein bound to the nickel loaded speharose. After the lysate had been loaded the column was washed with 8 volumes of binding buffer then 100mls of low imidazole buffer (20mM imidazole in binding buffer). The protein was eluted in high imidazole buffer (300mM imdiazole in binding buffer), which interferes with the binding of histidine with the sepharose causing the protein to be released from the column. The eluates and column washes were run on a 12% SDS-page gel and stained in Coomassie stain for 1hr at RT. The gel was then destained using Coomassie destain overnight and the eluate was checked for purity.

2.1.3.3 Purification of Non His-tagged Proteins

Protein was purified using immobilised metal ion affinity chromatography (IMAC) as previously described (Jones et al., 2004). As the prion protein has a high affinity for copper it can be purified using its metal binding site by loading chelating sepharose with copper, the prion protein will then bind to the sepharose and can be purified in this way. The IMAC column was packed with a bed of 6ml of chelating sepharose washed with 5 volumes of water, 0.3M copper sulphate and water. It was then saturated with binding buffer. After the lysate had been loaded the column was washed with binding buffer until protein was no longer coming off the column. The protein was eluted in high imidazole buffer (300mM imdazole in binding buffer). The eluate and column washes were run on a 12% SDS-page gel and stained in Coomassie stain for 1hr at RT. The gel was then destained using Coomassie destain overnight and the eluate was checked for purity.

2.1.3.4 Purification of the null mutants

A series of mutants were generated in our laboratories that had the histidines thought to bind metals replaced with alanines. These mutants included one that has all of its histidines removed (Zhu et al., 2008), the others had either the fifth site mutated or the octarepeat region histidines mutated into alanines (Klewpatinond et al., 2008). Mutants that did not contain the octarepeat region could not be purified by a column method and so were purified as previously described (Zhu et al., 2008). Briefly the proteins were lysed as previously described, however the pellet was not resuspended in binding buffer but underwent a series of washes as follows: The cell pellet was resuspended in a solution of lysis buffer containing 1% Igepal and 0.5% Triton X-100 by sonication at 30% for 1 minute. Protein was repelleted at 10,000 xg for 5 minutes, this step was repeated a further 5 times. The pellet was then resuspended, as before, in a further was solution containing 1% Triton X-100 and spun as before, this step was repeated 6 times. Finally the pellet was resuspended in a lysis buffer and spun as before, this was repeated 4 times to remove any detergent. The pellet was then resuspended in binding buffer, by sonication at 70% max power for 3x1 minute. Purity was confirmed by Coomassie stained SDS-PAGE gel.

2.1.3.5 Protein Refolding

Protein was bound to manganese as follows, protein was diluted in binding buffer to 1.5mg/ml, 1ml of this was then made up to 5mls with binding buffer, this was then concentrated to 1ml using a Vivaspin concentrator (15kDa cut-off), this removed the imidazole. The protein was then made up to 5mls using 5mM $MnSO_4$ in binding buffer; this was then concentrated back down to 1ml. The protein was then made up to 5mls with 5mM $MnSO_4$, this was added drop wise with stirring to prevent the protein precipitating out. This was spun down for 10 minutes at 4000rpm prior to concentrating to 1ml. This was then dialysed in MilliQ water to a 1 in 10,000 dilution in 12,000kDa cut-off dialysis tubing, which had previously been soaked in MilliQ water to ensure it was free of storage buffer (20% ETOH). The protein was then aged at –20C^o for 2 weeks prior to use.

Protein was refolded with water as follows, protein was diluted in binding buffer to 1.5mg/ml. The protein was then made up to 5ml with Milli Q water, this was added slowly with stirring to prevent precipitation. This was then centrifuged for 10 minutes at 4000rpm. This was then dialysed in Milli Q water to a 1 in 10,000 dilution in 12,000kDa cut-off dialysis tubing, which had previously been soaked in MilliQ water to ensure it was free of storage buffer (20% ETOH). The protein was then aged at –20C^o for 2 week prior to use.

2.1.4 Determination of Protein concentration

2.1.4.1 Bio-Rad protein assay.

The Bio-Rad protein assay is based on the Bradford assay, a colorimetric assay to quantify protein concentration. A series of standards were prepared using bovine serum albumin. The standards ranged in concentration from 0-1µg/ml. 1µl of each standard was added in triplicate to a 96 well plate. 1µl of each test sample was also added in triplicate with dilutions of sample, if necessary, to ensure the concentration fell within the standard curve. The dye concentrate was diluted 1:5 in Milli-Q water and 200µl was added to each well. The plate was incubated at room temperature for 5 minutes and then measured at 595nm in a plate reader. The resulting readings were plotted and the sample concentrations calculated.

2.1.4.2 Protein measurement by spectroscopy.

Protein was diluted 1:30 in the appropriate dilutant and the spectrophotometer was blanked using the dilutant. The sample was then measured from 200-900 nm so any contamination by DNA could be observed. The protein concentration was calculated using the OD_{280} this was multiplied by the dilution factor and divided by the extinction coefficient. All protein samples were measured using both methods to ensure an accurate reading.

2.2 Cell culture

2.2.1 Materials

All plastics were purchased from Fisher Scientific (Loughborough, UK)
Media was purchased from Lonza (Slough, UK)
OptiMeM serum free, phenol red free media (Invitrogen, Paisley, UK)
Penicillin purchased from Sigma-Aldrich (Poole, UK)
Streptomycin purchased from Sigma-Aldrich (Poole, UK)
Geneticin purchased from Gibco (Paisley, UK)
L-Glutamine purchased from Gibco (Paisley, UK)
Foetal Bovine Serum purchased from BioSera (East Sussex, UK)
Newborn calf serum purchased from Sigma-Aldrich (Poole, UK)
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega (Wisconsin, USA)
Phenazine methosulfate (PMS) from MP Biomedicals (California, USA)
Optimem media purchased from Gibco (Paisley, UK)
Fugene 6 from Roche diagnostics (Lewes, UK)
PD98059 from Promega (Wisconsin, USA)
DMSO purchased from Fisher Scientific (Loughborough, UK)
CM-H_2DCDFDA from Molecular Probes (Paisley, UK)
Bovine Serum Albumin purchased from Fisher Scientific (Loughborough, UK)
PMSF purchased from Sigma-Aldrich (Poole, UK)
Tris-HCl purchased from Sigma-Aldrich (Poole, UK)
Tris base purchased from Melford (Ipswich UK)
NaCl purchased from Fisher Scientific (Loughborough, UK)

Igepal CA-630 purchased from Sigma-Aldrich (Poole, UK)
SDS purchased from Fisher Scientific (Loughborough, UK)
EDTA purchased from Sigma-Aldrich (Poole, UK)
β-Mercaptoethanol purchased from Sigma-Aldrich (Poole, UK)
Complete Tablets purchased from Promega (Wisconsin, USA)
Sodium orthovanadate purchased from Sigma-Aldrich (Poole, UK)
Tween purchased from Sigma-Aldrich (Poole, UK)
PBS tablets purchased from Oxoid Ltd (Basingstoke, UK)
Glycine purchased from Fisher Scientific (Loughborough, UK)
HCl purchased from Fisher Scientific (Loughborough, UK)
Non-Fat dry milk powder (Marvel, UK)
Dulbeccos phosphate buffered saline purchased from Sigma-Aldrich (Poole, UK)
Poly-L-lysine purchased from Sigma-Aldrich (Poole, UK)
Multi-well borosilicate slides purchased from Fisher Scientific (Loughborough, UK)

2.2.2 Cell Maintenance

SMB cells are mesodermal cell line taken from a mouse clinically infected with the Chandler scrapie isolate, this cell line is capable of maintaining scrapie infection. SMB-PS cells are cells from the SMB cell line that have been successfully cured of scrapie using pentosan polysulphate (Birkett et al., 2001).

SMB and SMB-PS cells were maintained in 199 Medium of composition 1 g/L glucose, 2.2 g/L sodium bicarbonate, supplemented with 2mM L-Glutamine, 10% Foetal Bovine Serum (FBS) and 5% Newborn Calf Serum (NCS), with penicillin and streptomycin at final concentrations of 1U/ml and 0.5mg/ml respectively. Growth conditions were maintained at 37^{o}C, 5% CO_2 prior to any experiment.

F14 (PrP^{c} knockout mouse cerebellar-neuroblastoma fusion cells) and F21 ((PrP^{c} wild type mouse cerebellar-neuroblastoma fusion cells) were created in our laboratory by a fusion of cerebellar suspension from PrP null mice or WT mice with N18TG2 mouse neuroblastoma cells (Holme et al., 2003). F14 and F21 cells were maintained in Dulbeccos Modified Eagles Media (DMEM) comprised of 4.5 g/L glucose, 1.5 g/L sodium bicarbonate without sodium pyruvate and glutamine. This

was supplemented with 10% FBS and penicillin and streptomycin at final concentrations of 1U/ml and 0.5mg/ml respectively. Growth conditions were maintained at 37°C, 5% CO_2 prior to any experiment. All transfections were maintained using 0.5mg/ml geneticin.

N2a cells were established by from a spontaneous tumour of a strain A albino mouse (Klebe et al., 1970) and are a mouse neuroblastoma cell line. N2a cells were maintained in Earle's Minimal Essential Medium with Earle's BSS and adjusted to contain 1 g/L glucose, 2.2 g/L sodium bicarbonate, 2mM L-glutamine without sodium pyruvate. This was supplemented with 10% FBS, 0.1mM non essential amino acids and penicillin and streptomycin at final concentrations of 1U/ml and 0.5mg/ml respectively. Growth conditions were maintained at 37°C, 5% CO_2 prior to any experiment.

2.2.3 Transfection

2.2.3.1 PrP Constructs

A green fluorescent protein (GFP) fusion construct made using mouse PrP^c (accession number M13695), called pEGFP-C1-PrP(m) was donated to the lab by Marco Prado. Xenopus and turtle constructs were then created using mouse signalling sequences to ensure the correct expression of the proteins. The mouse construct contained the GFP sequence inserted at amino acid 42, the full mouse sequence is included in it. The Xenopus and turtle constructs contained the mouse sequence from amino acid 1 to 42, then the GFP and then the turtle or Xenopus sequence, the mouse sequence was also included at the C-terminus end of the protein from amino acid 231, again to allow correct processing of the protein. The construct was inserted into pEGFP-C1 vector (figure 2.1).

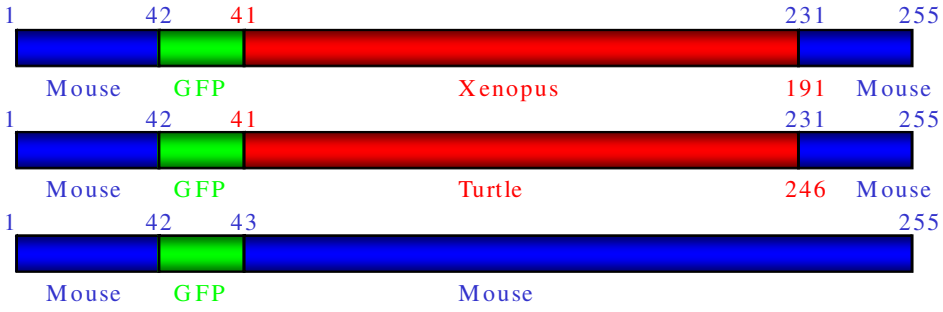


Figure 2.1: A schematic diagram of the chimeric constructs of PrP developed.

2.2.3.2 Promoter Constructs

The mouse PrP promoter construct was made by Lark Technologies on behalf of Dr Cathryn Haigh. The promoter construct was created using the mouse long incubation prion gene (accession number U29187). The construct was made in pd2-EGFP-1, a promoter-less vector containing destabilised GFP with a half-life of two hours. A construct containing the cytomegalovirus (CMV) promoter was also created. As the vector is CMV driven GFP expression was constitutive and could be used as a control for non-specific changes in fluorescence. The PrP promoter can be seen below (figure 2.2).

Figure 2.2: A schematic diagram of the mouse prion promoter construct used.

2.2.3.3 Stable Transfection

PrP-GFP constructs were stably transfected into F14 cells prior to toxicity assays. All transfections were performed using Fugene transfection reagent following the manufacturer's protocol. Briefly, cells were plated out at 50% confluence in 40mm dishes and left overnight to adhere prior to transfection. 3µl Fugene and 1-2 µg plasmid DNA were incubated at RT in 100µl serum free media for 20 minutes prior to transfection. The reagent was then added dropwise to the cells and the cells were incubated for 24h prior to selection. Transfected cells were selected using 100µg/ml of geneticin, once selection was complete the cells were maintained with 50µg/ml of geneticin. No stably transfected cells were used beyond passage ten.

2.2.3.4 Transient Transfection

F14 cells were transiently transfected with promoter constructs prior to promoter expression assessment using confocal microscopy. Chambered coverslips were coated in poly-L-lysine in order to ensure cell adhesion. Cells were plated out at 40-50% confluency and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. To 20µl of serum free DMEM, 0.6µl of Fugene was added and the tubes were tapped to mix, then 0.2ug of DNA was added. The mixture was incubated at room

temperature for 20 minutes and then added to the cells. The cells were not used for at least the first 24h after transfection to ensure a proper turnover of plasmid product.

2.2.4 Toxicity Assays

Toxicity assays were carried out to assess the toxicity of manganese refolded PrP and variety of manganese refolded PrP mutants. The toxicity of ApoPrP and the ApoPrP mutants was also investigated concurrently. These assays were carried out on F21 cells. The F21 cells were plated out at 75% confluency in DMEM and maintained at 37^{o}C, 5% CO_2 overnight, to allow the cells to adhere. The medium was then replaced with OptiMeM, a serum free medium without phenol red. The appropriate treatment was added to cells in triplicate and the cells were incubated for 48h prior to the MTS assay. Several assays investigating toxicity over time and the ability of anti-oxidants and non-mammalian prion proteins to rescue cells from MnPrP were also carried out. These used the method described above but used time-points ranging from 6h-72h.

The toxicity of MnPrP and ApoPrP to SMB and SMB-PS cells was also investigated. SMB and SMB-PS cells were plated out in media 199 at 90% confluency and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. The medium was then replaced with Optimem, a serum free medium without phenol red. The appropriate treatment was added to cells in triplicate and the cells were incubated for 48h prior to the MTS assay.

The toxicity of MnPrP to N2a cells was also investigated. N2a and cells were plated out in EMEM at 50% confluency and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. The medium was then replaced with Optimem, a serum free medium without phenol red. The appropriate treatment was added to cells in triplicate and the cells were incubated for 48h prior to the MTS assay.

The toxicity of the 106-126 peptide and a control peptide was also assessed in F21 cells and SMB cells. SMB cells were plated out in media 199 at 90% confluency and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. The F21 cells were plated out at 75% confluency in DMEM and maintained at 37^{o}C, 5% CO_2 overnight,

to allow the cells to adhere. In both cell lines the medium was then replaced with OptiMeM, a serum free medium without phenol red. The appropriate treatment was added to cells in triplicate to both cell lines concurrently and the cells were incubated for 48h prior to the MTS assay. The differing initial confluency that the cells were plated out at ensured that density at the start of the assay is comparable for each cell line.

2.2.5 MTS Assay

The MTS assay is a colorimetric method for determining viability. The assay is composed of MTS a tetrazolium salt and an electron coupling reagent PMS (phenazine methosulfate). MTS is reduced by cells into a formazan product. The MTS is converted into formazan by dehydrogenase enzymes only found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is therefore directly proportional to the number of living cells in culture.

All MTS assays were performed in 96 well plates with cell free wells to act as blanks, the assay was performed following the manufacturer's instructions. Cells (F21, SMB, SMB-PS or N2a) were plated and treated as described above. MTS was made up at 2 mg/ml in Dulbeccos phosphate buffered saline (DPBS) and PMS was made up at 0.96mg/ml in DPBS. 2mls of MTS and 100µl of PMS were mixed immediately prior to addition to cells, 20µl was added to each well and the cells were incubated in the dark at 37^{o}C for 1-4h and then read at 490nm on a spectrophotometer in order to determine cell viability.

2.2.6 Measurement of Reactive Oxygen Species (ROS)

Direct measurement of cellular reactive oxygen species was made using a 96 well plate assay using a fluorescent probe, CM-H_2DCDFDA. This probe is non-fluorescent until the acetate groups are removed by intercellular esterases and oxidation occurs within the cell when it emits a florescence that can be measured at excitation and emission of 488 and 534nm. As CM-H_2DCDFDA is cell permeable until oxidised it allows measurement of cellular production of ROS. Oxidation can

be induced by H_2O_2 (in the presence of endogenous metal ions), peroxynitrite, organic hydroperoxides and nitric oxide (Martin et al., 1998).

F21 or SMB cells were plated at 90-95% confluency and maintained at 37°C, 5% CO_2 overnight, to allow the cells to adhere. Cells were then treated with the PrP prior to the addition of the probe or the media removed and replaced by 50µl of 5µM probe in PBS (phosphate buffered saline). The cells were then incubated in the dark for 20 minutes. The probe was then removed from the cells and replaced by OptiMeM. If the cells had not already been treated the test reagent was added to 3 wells per experiment at this point. Fluorescence intensity was measured using a microplate reader with excitation and emission of 488 and 534nm respectively at time 0, 1h and 2h.

2.2.7 Confocal Analysis

F14 cells were plated out onto poly-L-lysine coated multi-well borosilicate slides at 50% confluency and left overnight to adhere. Cells were then transfected as previously described and left for 48h. Cells were treated as appropriate then incubated for 4h. Images of the cells were taken using the Zeiss LS 550 confocal microscope.

2.3 Cell signalling studies

2.3.1 Protein Extraction

In order to investigate cell signalling protein was extracted from SMB, SMB-PS and F21 cells for Western blotting. Cells from the appropriate cell line were plated out in order to achieve full confluency the following day in 24 well plates. Cells maintained at 37°C, 5% CO_2 overnight, to allow the cells to adhere. The cells were the then treated as appropriate prior to extraction. The extraction buffer was made up using RadioImmunoPrecipitation Assay (RIPA) buffer consisting of 50mM Tris HCl (pH 8), 150mM NaCl, 1% Igepal CA-630, 0.1% SDS and 1mM EDTA. A Complete tablet was added to the solution to provide inhibitors, 2µM sodium orthovandate and 1µM PMSF were also added.

30µl of extraction buffer was added to each well and incubated at 4°C for 20 minutes to allow the cells to lyse. The protein was stored at –80°C prior to use.

2.3.2 Western Blotting

Protein was denatured by boiling for 5 minutes in Laemmli's loading buffer (Laemmli, 1970), 20µg of protein was loaded on each well of a 12% SDS gel. The gel was run for 50 minutes at 35mA. The protein was transferred onto a nitrocellulose membrane using a Biorad semi-dry transfer apparatus.

The membrane was blocked for 1 hour using either 5% malted milk (MM) or 5% bovine serum albumin (BSA) in Tris Buffered Saline (40mM Tris base and 0.15M NaCl) with 1% Tween (TBST). The membrane was then incubated overnight, with rocking, in primary antibody at the appropriate concentration (see table 2.1). The membrane was then washed for 15 minutes, on the rocker, with three changes of TBST and incubated in secondary antibody for 1 hour, with rocking, at the appropriate concentration (table 2.1) and washed as described previously.

The membrane was then incubated with Millipore detection reagent for 5 minutes, any excess was then removed and the signal was visualised on X ray paper. Band intensity was quantified using Image J. Membranes were then stripped using a low pH stripping buffer (25mM glycine, 1%(w/v) SDS pH to 2 with concentrated HCl) as follows; the membranes were incubated in stripping buffer for 30 minutes with rocking. The membranes were then washed for 5 minutes in TBST with rocking. The membranes could then be re-probed with another antibody.

Primary Antibody	Concentration	Secondary Antibody	Concentration	Antibody dilutant
Tubulin	1:15000	Anti-Mouse	1:10000	1% MM in TBST
Actin	1:1000	Anti-Goat	1:2500	1% MM in TBST
Caspase 3	1:500	Anti-Rabbit	1:2500	5% MM in TBST
Caspase 9	1:1000	Anti-Rabbit	1:2500	5% MM in TBST
ERK	1:500	Anti-Rabbit	1:2500	1% MM in TBST
PERK	1:5000	Anti-Rabbit	1:2500	1% MM in TBST
p-p38	1:500	Anti-Mouse	1:2500	1% MM in TBST
p38	1:1000	Anti-Rabbit	1:2500	1% MM in TBST
BiP	1:1000	Anti-Mouse	1:2500	1% MM in TBST
p53	1:1000	Anti-Mouse	1:2500	1% MM in TBST
ICSM-18	1:10000	Anti-Mouse	1:10000	1% BSA in TBST
Caspase 12	1:1000	Anti-Rabbit	1:5000	1% MM in TBST
pAKT	1:1000	Anti-Rabbit	1:2500	1% MM in TBST
AKT	1:1000	Anti-Rabbit	1:2500	1% MM in TBST
14-3-3	1:1000	Anti-Rabbit	1:5000	1% MM in TBST

Table 2.1: A table showing all the primary antibodies used for western blotting. The concentration used the secondary used are also included. The antibody dilutant refers to the dilutant used for both the primary and secondary antibody.

2.3.3 Inhibitor Studies.

For inhibitor toxicity studies SMB and SMB-PS cells were plated out in a 96 well plate and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. The ERK inhibitor PD98059 was dissolved in DMSO prior to addition to the cells. Cells were treated with varying concentrations of PD98059 for 16h. Viability was assessed using an MTS assay. For the protein studies SMB and SMB-PS cells were plated out in a 24 well plate and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. The PD98059 was dissolved in DMSO prior to addition to the cells. Cells were treated with varying concentrations of PD98059 for 1h and pERK expression was assessed by western blotting.

For inhibitor toxicity studies, using a PKA inhibitor, SMB and SMB-PS cells were plated out in a 96 well plate and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. Cells were treated with varying concentrations of H89, PKA inhibitor for 24h. Viability was assessed using an MTS assay. For the protein studies SMB

and SMB-PS cells were plated out in a 24 well plate and maintained at 37^{o}C, 5% CO_2 overnight, to allow cells to adhere. Cells were treated with varying concentrations of H89 for 4h and pERK expression was assessed by western blotting.

2.4 Data Analysis

Intensity of measurements of live cell images was made using the Zeiss LSM software. Band intensity measurements for Western blots were made using Image J software, for all intensity reading a background measurement was subtracted from the values to eliminate any interference. All toxicity data was analysed using Microsoft Excel. One-way ANOVA was used for determining point differences on data points were considered to be significant once $P<0.05$ and $F>1$. A Students T-test was used for comparison of single points. A two tailed test assuming unequal variances was used. There was considered to be a significant difference once $P<0.05$.

3. Manganese and PrP Toxicity

Previous studies assessing the toxicity of PrP have used a variety of different models to investigate the mechanism of neuronal loss in prion disease. Some studies use PrP either purified from infected tissue or recombinant protein that has been misfolded or aggregated using a variety of methods including using SDS (Post et al., 2000; Stohr et al., 2008). More unusually transmembrane (Hegde et al., 1998) or cytosolic forms (Norstrom et al., 2007) of PrP have been used. Due to the difficulties in purifying large quantities of PrP^{sc}, many studies use synthetic peptides such as the well researched 106-126 peptide (Forloni et al., 1993) (Brown et al., 1994). However, it has been suggested that, as this peptide would not be found *in vivo* its use may not be an accurate representation of prion toxicity. Several studies have also used truncated forms of the prion protein, including C-terminus fragments with disease related mutations (Daniels et al., 2001).

Manganese has been hypothesized to be an important factor in prion disease, as studies have shown a perturbation of metal levels in sheep with scrapie and cows with BSE (Hesketh et al., 2007) and humans with CJD (Hesketh et al., 2008). The most striking result in these studies was an increase in manganese in certain areas of the brain and in the blood. Moreover, PrP can bind manganese, although the affinity of manganese was reported to be much lower than that of copper (Jackson et al., 2001). However recent work has shown that manganese binds to PrP with an affinity similar to that of several manganese binding proteins suggesting that the prion protein may be able to bind manganese *in vivo* (Brazier et al., 2008).

There is evidence that PrP bound to manganese displays PrP^{sc}-like qualities, including an increase in β-sheet content, increased PK (proteinase K) resistance and a tendency to aggregate (Brown et al., 2000). This has led to the hypothesis that PrP bound to manganese may be important in the disease pathogenesis. However, so far there has been no comprehensive study on the effect of manganese bound PrP on neuronal cells. Given the shared characteristics between MnPrP and PrP^{sc}, investigating MnPrP may further our understanding of prion disease. It has been established that prion disease leads to neuronal cell death, which is thought to be

caused by the toxicity of PrP^{sc}. Therefore the first part of this study intends to characterise the toxicity of the protein to neuronal-like cells. We intended to investigate the toxicity of MnPrP using neuronal cell lines. The cell lines used were the F21 cell line, a neuroblastoma fusion cell line and the N2a cell line, a well characterised neuroblastoma cell line that has previously been shown to be susceptible to PrP^{sc} toxicity and infection. We showed MnPrP was toxic to both these cell lines. The results also confirmed the use of MnPrP as a suitable model of toxicity for use in further studies.

One of the puzzles of prion research is the ability of cells infected with scrapie to carry infection through repeated passages without any loss of cell viability. This is in contrast to animal models of mice and sheep infected with scrapie, where the animals suffer massive neuronal loss after infection (Fairbairn et al., 1994; Giese et al., 1995). We investigated toxicity in SMB and SMB-PS cell lines. These cell lines were originally derived from a mouse infected with the Chandler strain of scrapie (Clarke and Haig, 1970), the SMB-PS cell line has been successfully cured of scrapie using pentosan sulphate (Birkett et al., 2001). The results demonstrated that MnPrP is toxic to both SMB and SMB-PS cells and that, as in the case of PrP^{sc}, this cell death is due to apoptosis. We hypothesise that cells infected with scrapie may be able to survive as the levels of PrP^{sc} produced by the cells may not be sufficient to cause toxicity.

3.1 Purification of PrP

Full-length mouse PrP (amino acids 23-231) was cloned into a pET23a vector with a 6 histidine tag on the C-terminal end of the protein to allow purification. The protein was transformed into *E Coli* and expression was induced using IPTG. The protein was purified using nickel chelating sepharose IMAC column. The resulting protein was then separated on a SDS-PAGE gel and stained with coomassie to establish purity (figure 3.1a) and Western blotting was used to confirm it was mouse PrP (figure 3.1b)

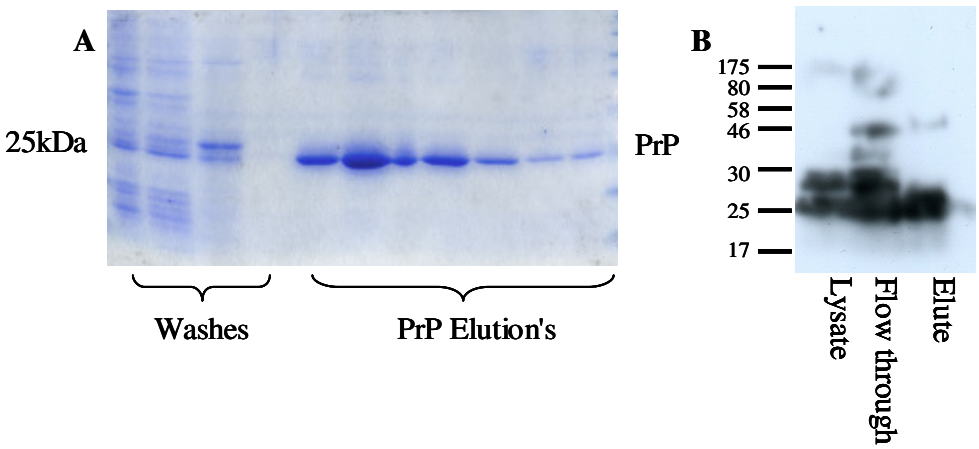


Figure 3.1: Representative blots of purified WT recombinant PrP. A: A representative gel of column washes and protein eluate, 20µl was loaded into each lane of a 12% SDS gel. The gel was then stained and the purity of the eluate was assessed. B: A representative western blot using the PrP specific antibody ICSM-18 showing washes and an elution

PrP without a 6 his-tag was also used for certain experiments. This construct was purified using one of two methods, either using a copper chelating sepharose column (figure 3.2) or using a wash method. With both methods, the purity of the protein was assessed by comassie staining.

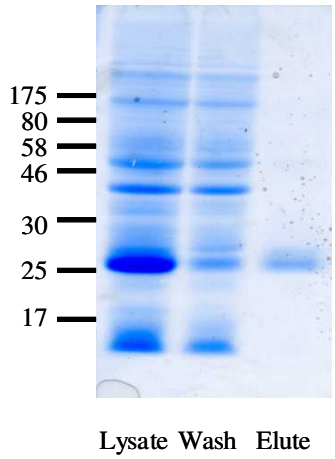


Figure 3.2: Representative gel of purified non-his-tagged PrP. A: A representative gel of column washes and protein eluate from a copper column, 20µl was loaded into each lane of a 12% SDS gel. The gel was then stained and the purity of the eluate was assessed.

The purified protein was refolded in the presence of manganese as previously described in (Brown et al., 2000) prior to extensive dialysis to remove any excess manganese. Mass spectrometry analysis revealed that the full length protein was able to bind two atoms of manganese (Uppington and Brown, 2008).

3.2 Toxicity of Manganese bound PrP (MnPrP)

Work had assessed the toxicity of MnPrP on primary neuronal cultures (Uppington and Brown, 2008), as primary neurones expressing PrP^{c} have been shown to be susceptible to PrP^{sc}. In order to test MnPrP toxicity, cultures of cerebellar granule neurons (CGNs) from wild-type and PrP knockout mice were prepared and treated with either MnPrP or apoPrP (water refolded PrP) for 48h. Viability was then assessed using a MTT cell proliferation assay. The MTS assay is a colorimetric method for determining viability.

The MnPrP was significantly more toxic to CGNs than apoPrP, however there was no difference in toxicity between MnPrP and apoPrP in PrP-knockout cultures (figures 3.3). When the toxicity of MnPrP in knockout cell lines was compared to the toxicity of MnPrP in WT cells there was a significant increase in toxicity in WT cells. This suggests that cellular PrP expression is required for MnPrP induced toxicity.

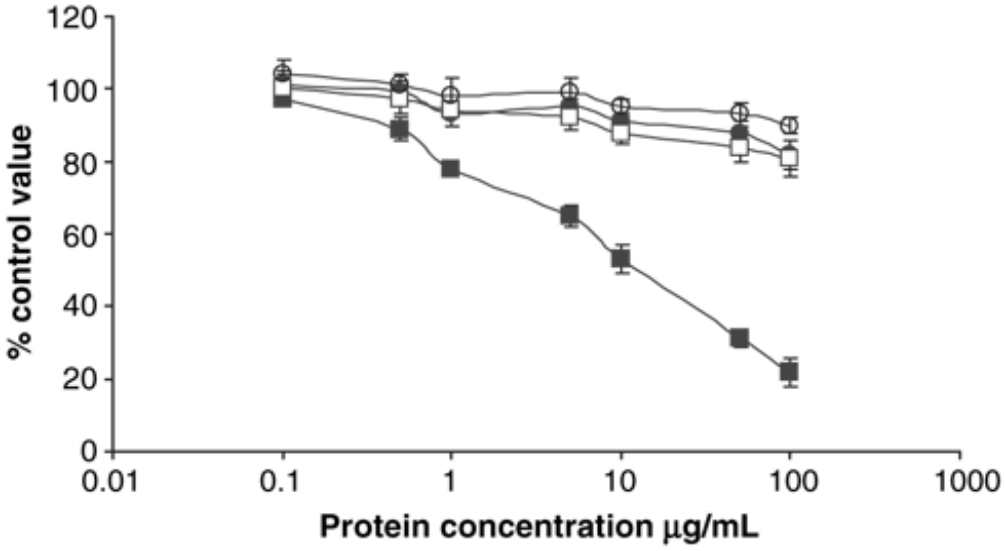


Figure 3.3:Toxicity of MnPrP to primary CGNs. Primary cultures of WT (squares) or KO-PrP (circles) CGNs were treated for 48h with varying concentrations of MnPrP (Black symbols) or apoPrP (open symbols). Survival was assessed using an MTT assay. MnPrP was significantly toxic to cells at dosages above 1µg/ml as assessed by students T-test. Error bars are SEM each point represents at least 4 repeats.

To exclude the possibility that the toxicity observed was due to the manganese present in the protein we tested the toxicity of $MnSO_4$ to cells. Cells were treated for 48h with varying concentrations of $MnSO_4$ and cell viability was assessed by an MTS assay, MTS is soluble and in the presence of mitochondrial activity it undergoes a colour change, allowing a quantitative analysis of cell viability. Significant cell death was observed at doses above 10µM. Mass spectroscopy analysis revealed that MnPrP binds two manganese atoms per molecule, implying that treatment of cells with up 125µg/ml MnPrP would not result in manganese toxicity in cells treated for 48h (figure 3.4). Further experiments have revealed that doses up to 175µg/ml were not toxic to F21 cells (Uppington and Brown, 2008).

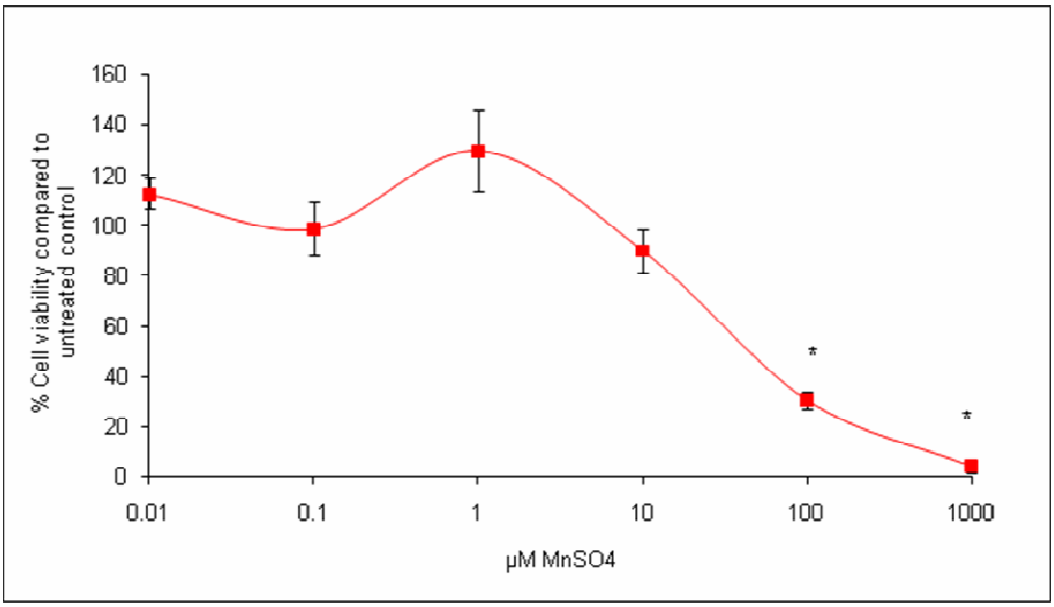


Figure 3.4: Manganese toxicity to F21 cells. F21 cells were treated for 48h with increasing concentrations of $MnSO_4$. Cell viability was then assessed using an MTS assay, a cell proliferation assay. $MnSO_4$ was found not to be toxic to cells up to a concentration of 10µM (p=0.55 as tested by students T test). Graph depicts cell viability compared to control. N=6 (Error bars are SEM) * Shows significant points.

In order to further develop a model of toxicity we choose to use neuroblastoma cell lines. We investigated MnPrP toxicity in F21 cells a fusion neuroblastoma cell line that has been shown to express the neuronal marker including Neu-N, and express PrP. Toxicity was also investigated in the well characterised N2A cell line, a neuroblastoma cell line that has been shown to express PrP.

We treated F21 cells with a variety of concentrations of MnPrP, and assessed toxicity over a range of time points. In order to assess viability we used an MTS assay. No significant cell death was seen at 6h, however by 24h significant cell death was seen at a dose of 40µg/ml and this was repeated at 48h. A significant increase in death between 24 and 48h was also observed when comparing the 40µg/ml dose. By 72h, there appeared to be a slight decrease in cell death, although this was not significant (figure 3.5).

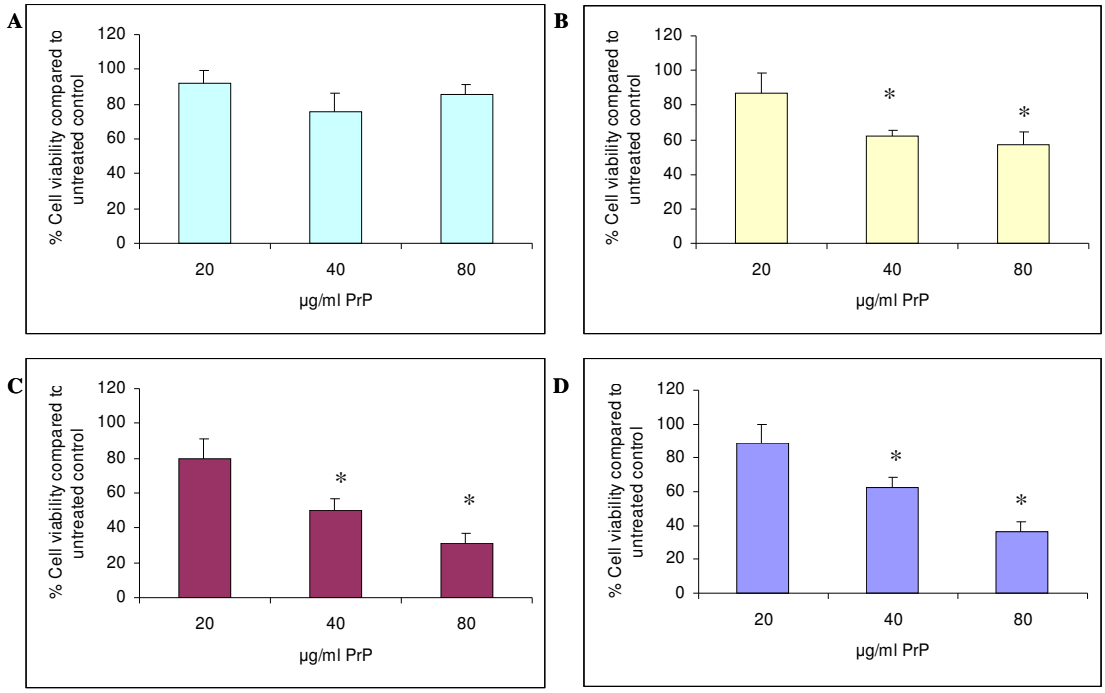


Figure 3.5: Toxicity of MnPrP over time. F21 cells were treated with increasing concentrations of MnPrP for either 6h (A), 24h (B), 48h (C) or 72h (D). Cell viability was then assessed using an MTS assay. N=4 (Error bars are SEM). * Significant points were assessed by Students T-test.

Having established the toxicity of MnPrP in F21 cells we tested the toxicity of both MnPrP and apoPrP (water refolded PrP) to F21 cells. F21 cells were treated with MnPrP or apoPrP for 48h and cell viability was assessed by MTS assay. MnPrP was found to be significantly more toxic to F21 cells than apoPrP, leading us to the conclusion manganese binding to PrP is important in toxicity, although unlike the results observed in primary neuronal cultures apoPrP was toxic to F21 cells (figure 3.6)

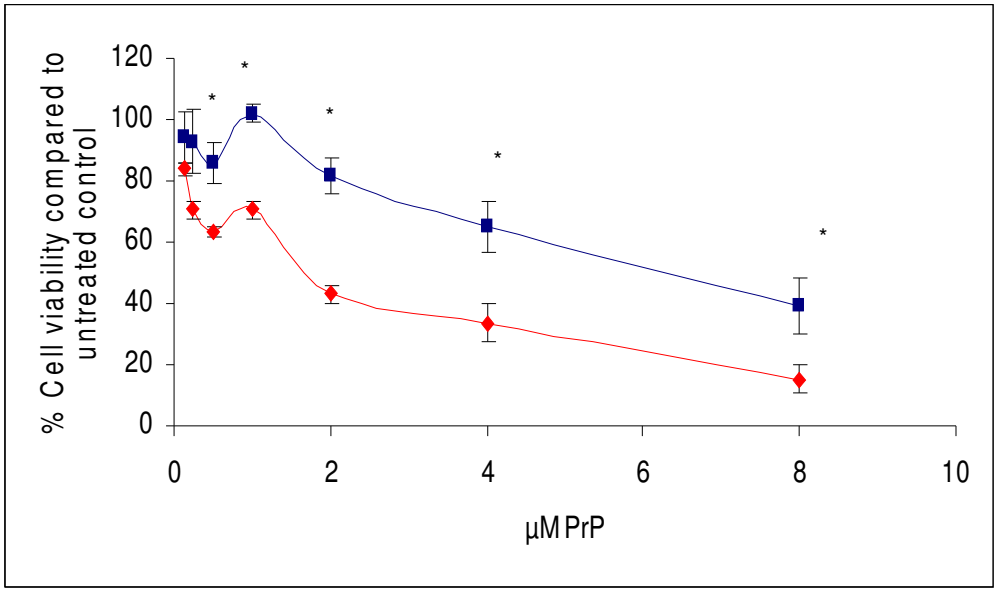


Figure 3.6: Toxicity of MnPrP and apoPrP to F21 cells. F21 cells were treated for 48h with increasing concentrations of either MnPrP (red line) or apoPrP (Green line). Cell viability was then assessed using an MTS assay. Graph depicts cell viability compared to control. N=10 (Error bars are SEM). Significance was assessed using a one-way ANOVA $p<0.05$.

To ensure the toxicity observed in F21 cells was reproducible in other cell lines we investigated the toxicity of MnPrP in N2A cells. The N2a cell line is a well established neuronal cell line that has been shown to be susceptible to both infection (Butler et al., 1988) and PrP toxicity (Hetz et al., 2003b). F21 and N2a cells were treated for 48h with MnPrP, and viability was assessed using a MTS assay. Both F21 and N2a cells were shown to respond in a similar dose dependant manner to MnPrP (figure 3.7). The levels of manganese added to the N2a cells were are not significantly toxic to the cells (data not shown).

We also investigated the effect of MnPrP on a knockout cell line. F14 cells were treated for 48h with MnPrP and viability was assessed using an MTS assay. There was significant increase in cell death observed in WT cells when compared to knock-out cells (figure 3.8).

We have shown that MnPrP is toxic to cell lines, although a higher concentration is required to induce cell death than observed in primary cultures, we were also able to show that cell lines that do not express PrP are not susceptible to MnPrP toxicity. Finally we demonstrated that apoPrP was less toxic than MnPrP.

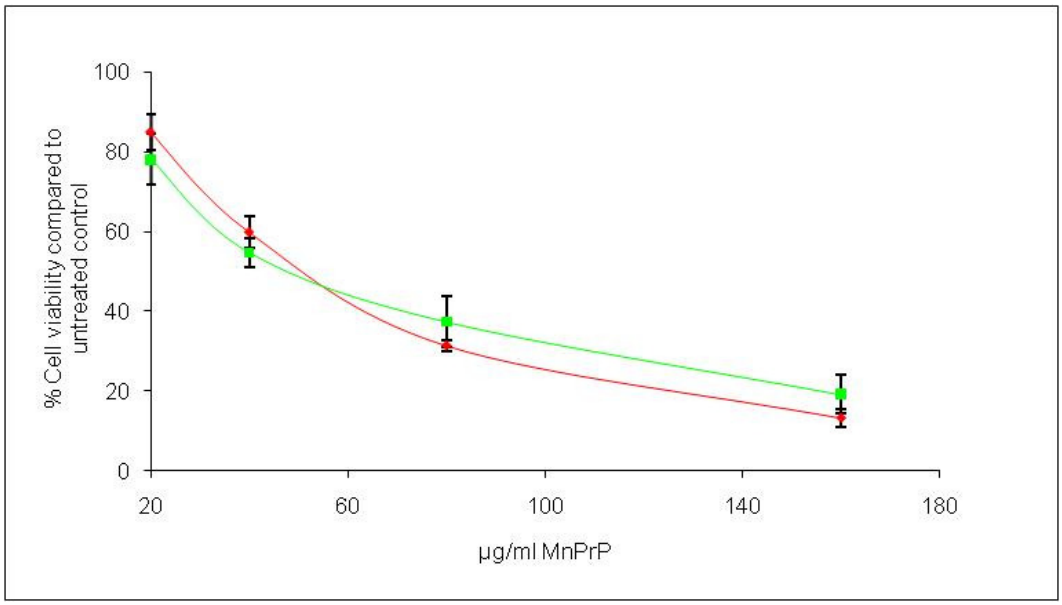


Figure 3.7: Toxicity of MnPrP to N2a cells. F21 (red line) and N2a (Green line) cells were treated for 48h with increasing concentrations of MnPrP. Cell viability was then assessed using an MTS assay, N=6 (Error bars are SEM). No points were significant as determined by one-way ANOVA.

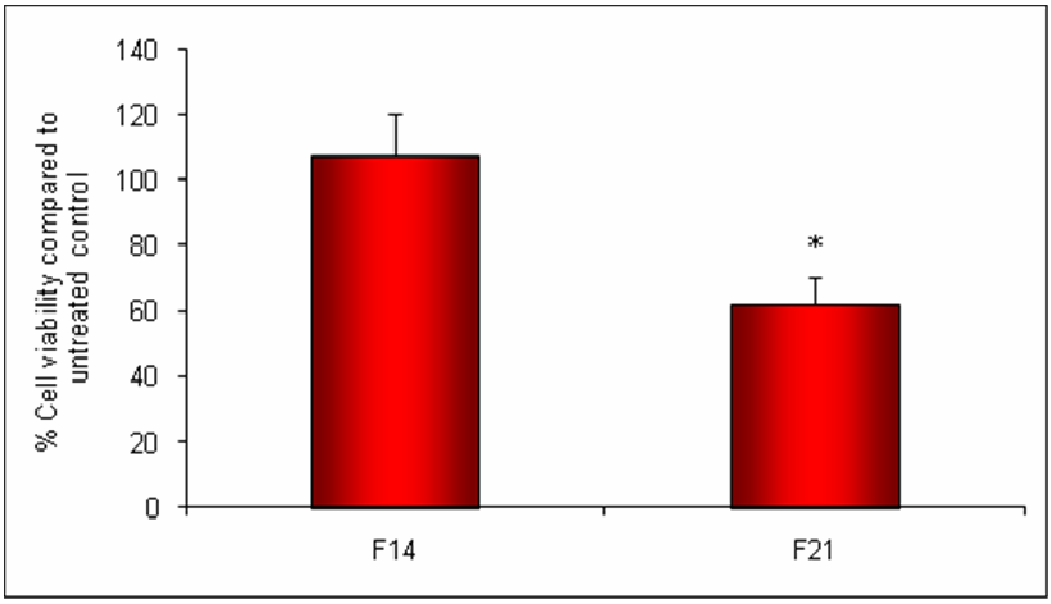


Figure 3.8: The toxicity of MnPrP to PrP null cells. F14 and F21 cells were treated for 48h with 80µg/ml of MnPrP. Cell viability was then assessed using an MTS assay, N=3(Error bars are SEM). P<0.05 as tested by Students T-Test.

Previous reports have suggested that when PrP is initially bound to manganese, it displays characteristics similar to PrP bound to copper, however, if the protein is aged for 2 weeks it becomes more protease resistant and there is an increase in β-sheet structure (Brown et al., 2000). In order to investigate whether aging was

important in the toxicity of the protein we treated cells with aged or un-aged protein. Protein was bound to manganese as previously described and then allowed to age at –20°C for two weeks. This was compared to fresh MnPrP made immediately prior to the experiment. F21 cells were incubated with 40 µg/ml of MnPrP for 48h and toxicity was assessed using an MTS assay. We found that aged MnPrP was significantly more toxic to F21 cells than fresh PrP (figure 3. 9) this suggests that the ageing process not only changes the physical properties of MnPrP but increases its toxicity to cells.

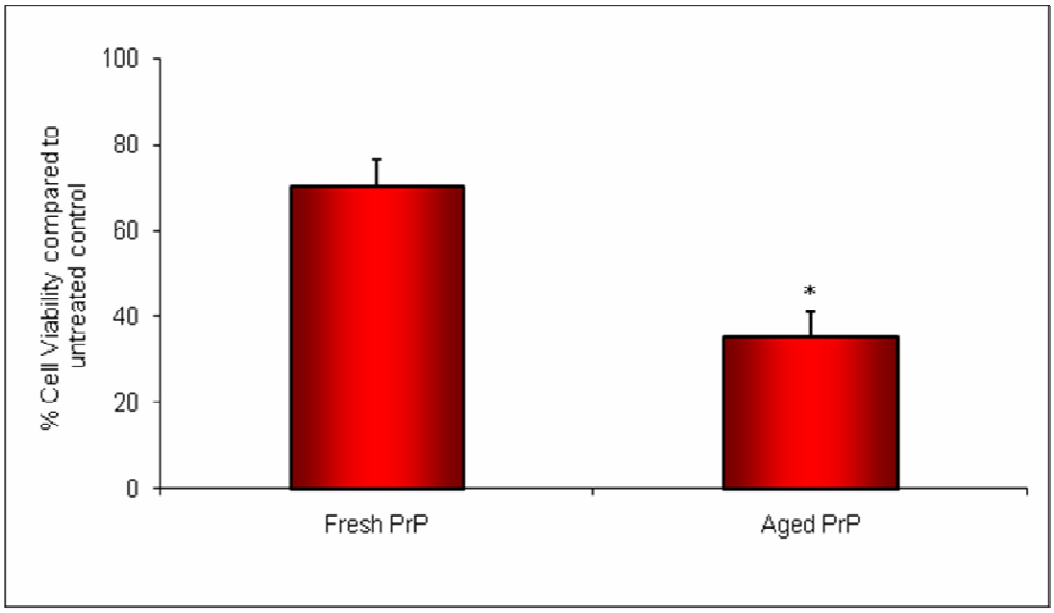


Figure 3.9: The toxicity of aged and un-aged MnPrP. The toxicity of manganese PrP either fresh or aged for 2 weeks at –20°C was assessed using an MTS assay. Prior to the assay cells were treated with 40µg/ml MnPrP for 48h. Error bars are SEM. Each bar represents at least 5 repeats. P<0.01 as assessed by students T-Test.

In order to ensure that MnPrP was a sound model of PrP toxicity we wanted to ensure all excess manganese was removed from the protein, as the excess manganese could be toxic to the cells. We also wanted to investigate the survival of the protein in culture, as survival in culture may play a role in toxicity, and finally we wanted to investigate whether the his-tag played any role in MnPrP toxicity.

Extensive dialysis is used to remove any excess manganese from MnPrP prior to its addition to cells. Previous work by (Brown et al., 2000) had used MnPrP that had been aged and then dialysed, however we decided to perform dialyse before aging, in order to ensure the complete removal of any excess manganese. F21 cells were

treated for 48h with 80 µg/ml MnPrP. We found that there was a significant increase in toxicity in cells treated with PrP that had been dialysed before aging however this difference was small (figure 3.10).

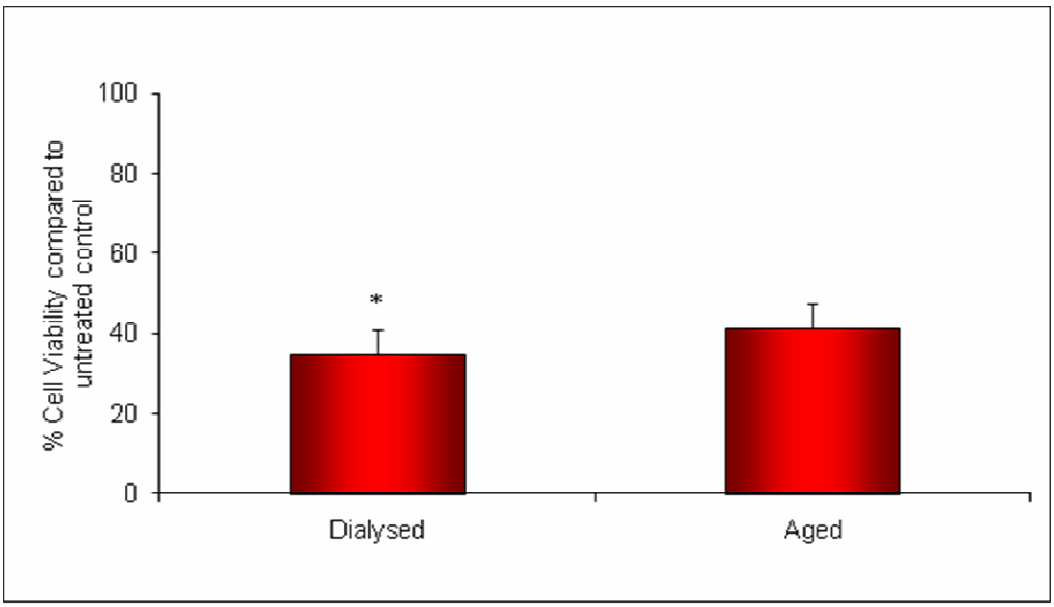


Figure 3.10: The effect of dialysis on the toxicity of MnPrP. Viability of F21 cells incubated for 48h with 80µg/ml MnPrP either, dialysed and aged for 2 weeks (manganese dialysed) or aged for 2 weeks and then dialysed (manganese aged), as assessed by MTS assay. Graphs show viability compared to control. (Error bars are SEM) Each bar represents at least 6 repeats. P<0.01 as assessed by students T-Test.

To exclude differences in toxicity observed between apoPrP and MnPrP being due to apoPrP degrading more quickly in culture, the ability of MnPrP and apoPrP to survive in culture was tested. MnPrP or apoPrP was incubated at 37^{o}C in media with or without cells for at least four days. We found that apoPrP was able to survive in culture for 4 days. Interestingly, we found that apoPrP was cleaved in the presence of F21 cells giving a product of about 16kDa (figure 3.11). MnPrP was also able to survive in culture for up to 4 days and was also cleaved in the presence of cells (data not shown).

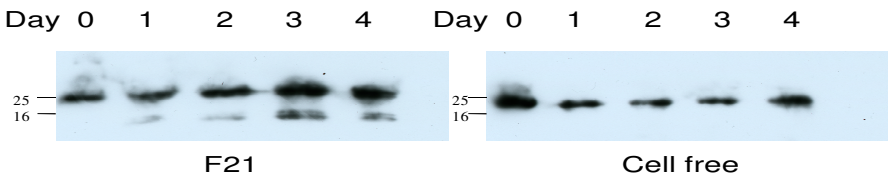


Figure 3.11: The ability of apoPrP to survive in culture. 80µg/ml apoPrP was incubated at 37^{0}C either media alone or with F21 cells and the media was removed for analysis. 20µl of media was separated on a 12% SDS-PAGE gel. The gel was transferred onto nitrocellulose and western blotted with ICMS-18 a monoclonal antibody against PrP.

As the protein we were using was his-tagged we investigated whether the his-tag had any influence on the toxicity of the MnPrP. There was no significant difference in toxicity between the tagged and untagged protein suggesting that the his-tag plays no part in MnPrP toxicity (figure 3.12a). The toxicity of recombinant untagged PrP was also investigated was found to be significantly less toxic than MnPrP (figure 3.12b).

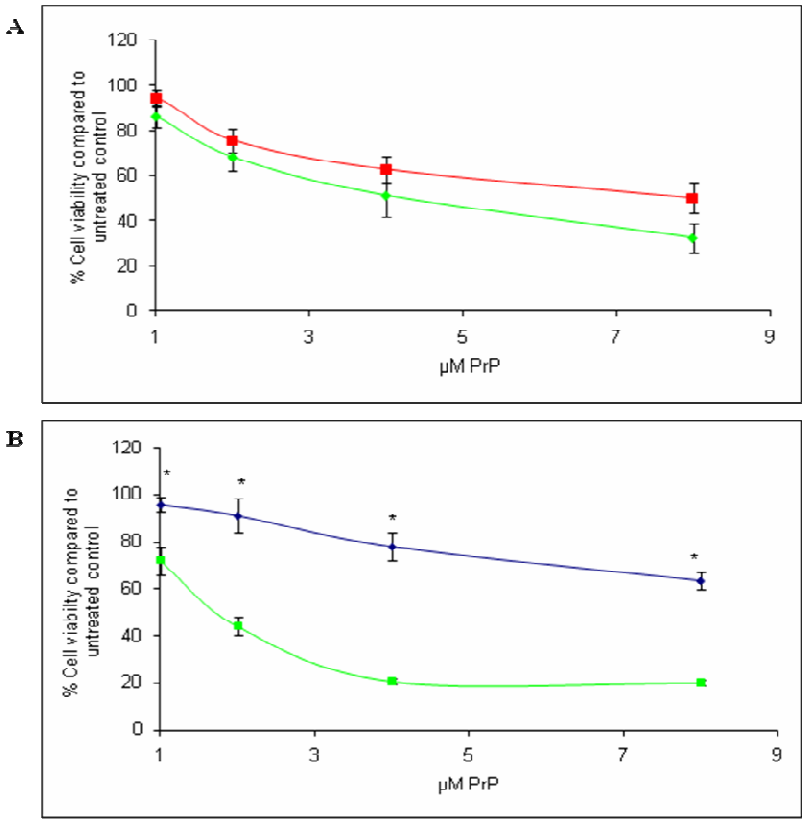


Figure 3.12: The toxicity of non-his-tagged PrP. A: F21 cells were treated for 48h with either untagged WT MnPrP (red line) or tagged MnPrP (green line). Cell viability was then assessed using an MTS assay (error bars are SEM.) B: F21 cells were treated for 48h with either untagged apoPrP (blue line) or untagged MnPrP (green line). Cell viability was then assessed using an MTS assay. (Error bars are SEM.) Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

3.3 Properties of MnPrP.

We have also shown that aged MnPrP is partially resistant to proteinase K. MnPrP was incubated with varying concentrations of PK for 30 minutes. MnPrP showed some level of PK resistance at all concentrations tested, which is in direct contrast to apoPrP which was fully digested by all but the lowest concentration of PK (figure 3.13).

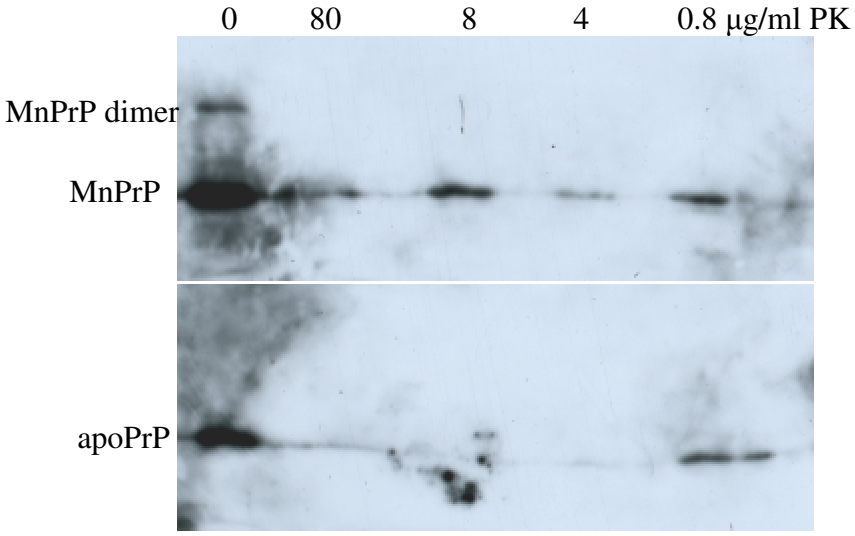


Figure 3.13: The PK resistance of MnPrP and apoPrP. MnPrP and apoPrP were treated for 30 minutes with varying concentrations of PK. The protein was then separated on a 12% SDS-PAGE gel and Western blotted using ICMS-18, a specific monoclonal PrP antibody.

3.4 Cell death induced by MnPrP in infected cells

The toxicity of MnPrP was also investigated in infected cells. The SMB cell line, a cell line persistently infected with scrapie and SMB-PS cells, a cell line cured using pentosan sulphate, were used. The SMB cell line, like several other scrapie infected cell lines has been shown to survive with the infection. Cells were treated for 48h with varying concentration of his-tagged MnPrP and viability was assessed using an MTS assay. His-tagged PrP was used for the remainder of experiments in this thesis unless otherwise stated.

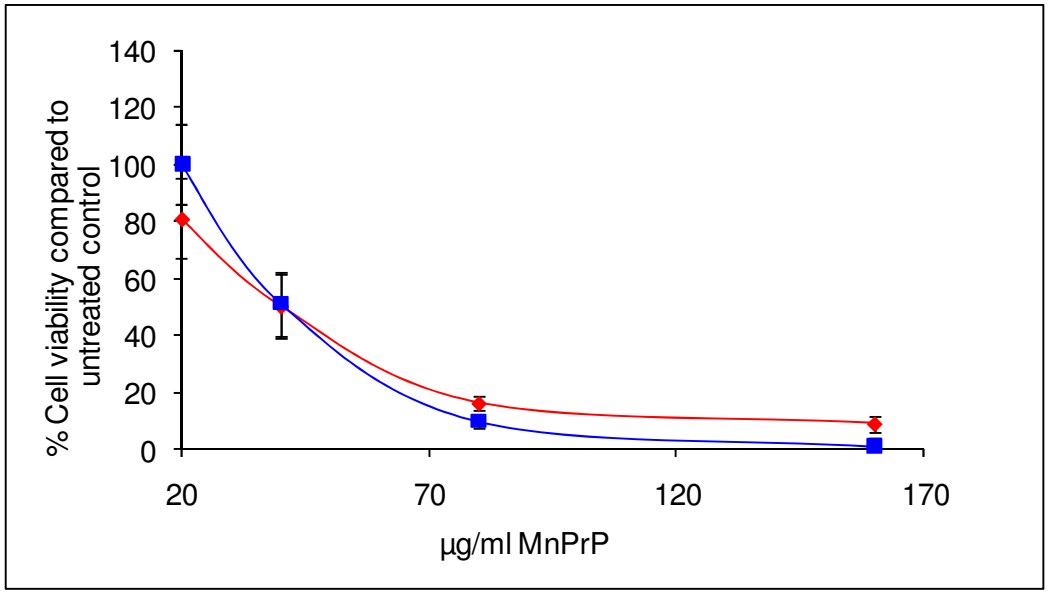


Figure 3.14: Toxicity of MnPrP to scrapie infected and cured cells. SMB (red line) or SMB-PS (blue line) cells were treated for 48h with increasing concentrations of MnPrP. Cell viability was then assessed using an MTS assay, a cell proliferation assay. Graph depicts cell viability compared to control. N=3 (Error bars are SEM)

Cells died in similar manner to F21 and N2A cells suggesting resistance to scrapie infection did not prevent cell death mediated by MnPrP (figure 3.14).

In order to understand the mechanism of cell death we investigated whether apoptotic signals were activated in response to MnPrP. To confirm that MnPrP caused apoptosis, we investigated whether MnPrP caused caspase 3 cleavage in SMB cells. Several previous reports have shown caspase 3 activation both in cells treated with the 106-126 peptide (Thellung et al., 2002) and in the brains of mice infected with scrapie (Jamieson et al., 2001). Caspase 3 activation is one of the final steps in the induction in apoptosis. Cells were treated for 4, 8, 16 and 24h with 80µg/ml MnPrP and caspase 3 cleavage was assessed using Western blotting. Caspase 3 cleavage was seen after 16h of treatment with MnPrP (figure 3.15).

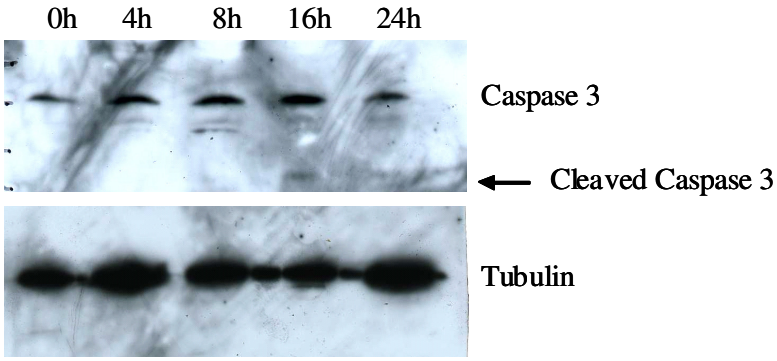


Figure 3.15: Caspase 3 cleavage in infected cells treated with MnPrP. SMB cells were treated with 80µg/ml PrP for varying lengths of time. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. Caspase 3 expression and activation was assessed using western blotting. Lane 1: 0h, Lane 2: 4h, Lane 3: 8h, Lane 5: 16h, Lane 6: 24h.

To investigate this phenomenon further we treated both SMB and F21 cells with the 106-126 peptide and the octarepeat peptide (amino acids 51-90). Both F21 cells and SMB cells were sensitive to treatment with the peptide (figure 3.16) confirming that SMB cells are sensitive to PrP toxicity despite resistance to scrapie infection.

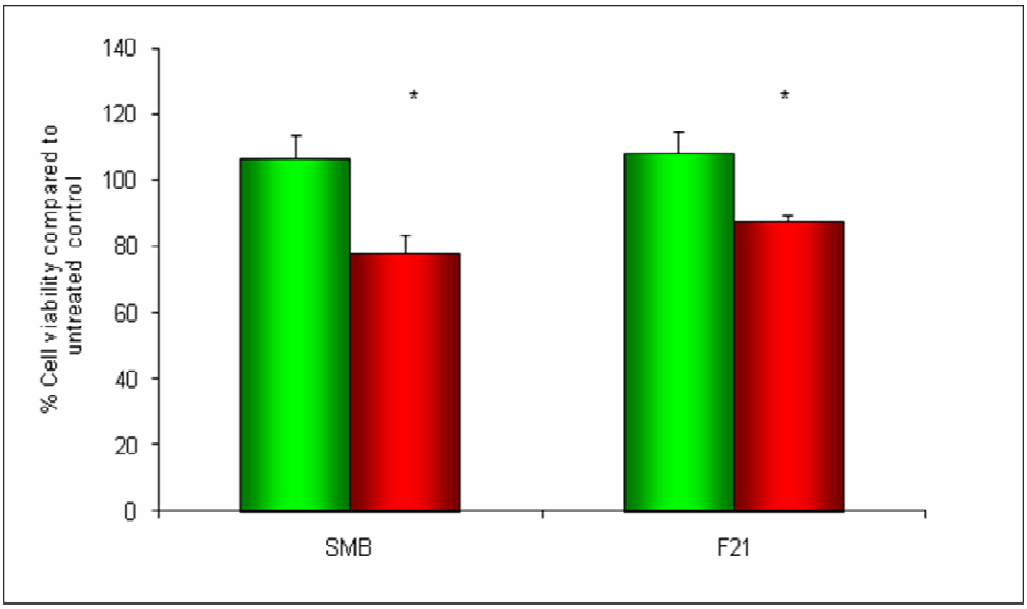


Figure 3.16: Toxicity of 106-126 to both F21 cells and scrapie infected cells. Cells were treated for 48h with increasing concentrations either 100 µM control peptide (green) or 106-126 (red). Cell viability was then assessed using an MTS. Graph depicts cell viability compared to control. (Error bars are SEM) n=4. Significant cell death was seen in cells treated with 106-126 peptide when compared to cells treated with the control peptide in both F21 and SMB cells as tested by students T-Test p>0.01.

We hypothesised that the infection in SMB cells might produce low levels of PrP^{sc} that allow the cells to survive infection. To investigate this we treated the both F21 and SMB cells with PK digested SMB lysate for 24h prior to the addition of MTS, no evidence of cell death was observed even at the highest concentration of lysate. This may be due to the low levels of PrP^{sc} expressed by SMB cells. Previous work has shown that infected cultures may only contain as few as 1% infected cells although in certain cells lines this can increase to 13% (Nishida et al., 2000; Race et al., 1987). Work in our lab has also shown that over expression of PrP in SMBs by stable transfection is not possible for more than a few passages suggesting cells with more PrP^{c} available to convert to PrP^{sc} either divide slower or are unable to survive (unpublished data) (figure 3.17).

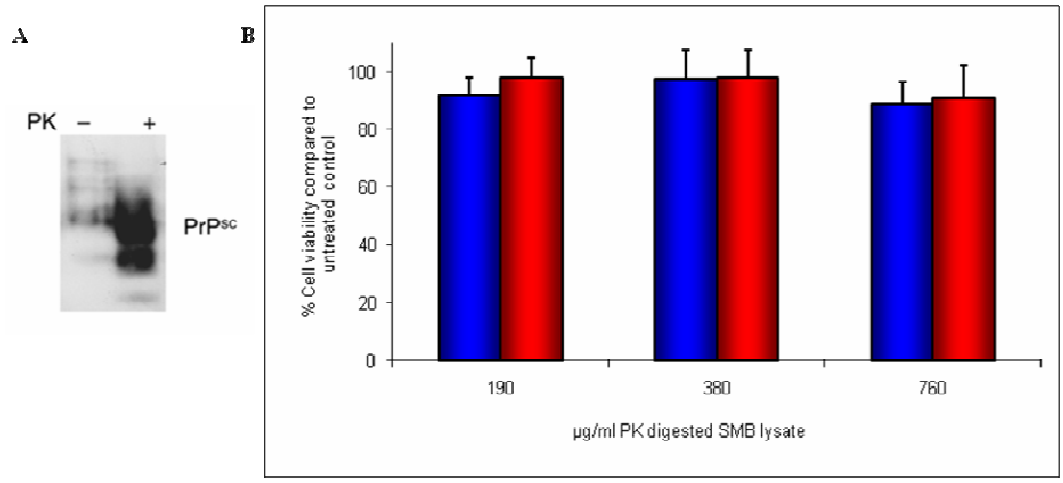


Figure 3.17: Toxicity of SMB cell lysate. A: SMB cell lysates were extracted and digested with PK the resulting digest was then concentrated and Western blotted using a PrP specific antibody ICSM-18 an un-concentrated un-digested sample was also run. B: SMB cells (blue) or F21 cells (red) were treated for 48h with increasing concentrations of PK digested SMB lysate. Cell viability was then assessed using an MTS assay, a cell proliferation assay. Graph depicts cell viability compared to control. N=4 (Error bars are SEM).

3.5 Discussion

There has been extensive research into prion diseases, most of it using models. Work using mouse models has revealed that the disease is caused by a novel infectious protein termed a prion (Prusiner, 1982). Work in mouse models also demonstrated that the propagation of PrP^{sc} and hence the disease requires cellular PrP expression (Brandner et al., 1996; Bueler et al., 1993; Prusiner et al., 1993; Weissmann et al., 1994). The level of expression of PrP^{c} can affect the incubation time of the disease, but not the final pathology. Over-expression results in a shorter disease incubation time (Fischer et al., 1996) and lower PrP^{c} expression results in a longer incubation period (Bueler et al., 1994).

It has been demonstrated that the massive neuronal loss observed in prion disease is due to neuronal apoptosis (Fairbairn et al., 1994; Giese et al., 1995), although the signalling pathway leading to apoptosis remains unclear. Work on animal models also revealed a species barrier, transmission of classical CJD to mice is difficult and often fails, however transmission to transgenic mice expressing only the human prion protein lacks a species barrier (Collinge et al., 1995b). Mouse models also provided the first evidence that PrP^{sc} assembles into filaments within the brain and that these filaments accumulate in extracellular spaces to form amyloid plaques (DeArmond et al., 1985). Animal models can provide a wealth of information on prion diseases however, they are expensive and cell culture models often provide more information at the cellular level.

Cell culture models using PrP^{sc} purified from infected brains replicate many of the properties of prion diseases observed *in vivo*. PrP^{sc} is toxic to neuronal cells *in vitro* (Post et al., 2000), and cell death is due to apoptosis (Hetz et al., 2003b). However the difficulty of purifying large quantities of PrPsc has meant that alternate models of prion disease have been developed.

Much early work used peptides, and they are still widely used as a model of prion toxicity today. Perhaps the most widely used is a short peptide sequence of human PrP encompassing the amino acids 106-126. This region has several of the properties

associated with PrP^{sc} including the tendency to aggregate (Forloni et al., 1993) and a high level of β-sheet structure (Selvaggini et al., 1993). The 106-126 peptide is selectively toxic to PrP expressing neurones (Brown et al., 1994), however the validity of using the peptide has been called into question as although this sequence is present in several longer peptides isolated from cerebral amyloid plaques of patients suffering from GSS (Ghetti et al., 1996), the PrP 106–126 peptide has never been found *in vivo*.

There have been increasing numbers of studies that use recombinant PrP that has been converted into misfolded, aggregated, and protease resistant forms like PrP^{sc} using chemicals such as SDS (Stohr et al., 2008) or exposure to harsh denaturants and shaking (Novitskaya et al., 2006). These chemicals would not be found at these concentrations in tissue and as such do not represent a conversion that could take place *in vivo*. Several studies have used truncated versions of the prion protein, such as the 90-231 fragment rather than using the full-length protein (Corsaro et al., 2006; Thellung et al., 2007).

Previous studies have demonstrated that amyloid fibrils of recombinant PrP are toxic to both primary neuronal cells and cell lines. Down-regulation of PrP^{c} expression resulted in a decrease in toxicity (Novitskaya et al., 2006). This suggests that toxicity requires cellular PrP expression. Full-length recombinant PrP that has an α-helical structure and is not aggregated has been shown not to be toxic to neuronal cells (Novitskaya et al., 2006) suggesting structure is important for toxicity. Studies using a thermally denatured fragment of PrP have also shown that the conversion to a β-sheet structure from a α-helical structure results in an increase in toxicity (Corsaro et al., 2006).

Studies using recombinant PrP have demonstrated its usefulness as model of prion disease, as it replicates several of the hallmarks of prion disease, it causes neuronal apoptosis, once converted into an amyloid form and toxicity requires cellular prion expression. As recombinant PrP has been shown to be a suitable model of prion disease, we choose to use it in this study. PrP bound to manganese has been shown to demonstrate several of the properties of PrP^{sc} (Brown et al., 2000), however there had been limited studies investigating the toxicity of MnPrP.

Our study has shown that MnPrP is toxic to neuronal cells and is selectively toxic to PrP-expressing cells. Toxicity was dependent on manganese binding to PrP in primary cultures and significantly increased its toxicity to F21 cells, suggesting that manganese binding plays an important role in prion toxicity in our model. We also showed that MnPrP was partially resistant to PK digestion. Previous work had shown that MnPrP contained increased levels of β-sheet structure, which has been shown to be important for toxicity, and an intact disulphide bridge (Brown et al., 2000; Wong et al., 2001b). Thus MnPrP reproduces many of the characteristics of PrP^{sc} and we suggest it can be used as a valid model of prion toxicity *in vitro*.

3.5.1 The Prion Protein and Metal Binding

Our model uses aberrant metal binding to covert the recombinant PrP to a more toxic form. Metal binding is thought to be important for the function of the prion protein and copper binding has been suggested to provide structure in the relatively unstructured N-terminal region of the prion protein (Jones et al., 2004) as well as effecting the structure of the C-terminal region (Zhu et al., 2008). The prion protein has been shown to bind copper *in vivo* (Brown et al., 1997a). It has also been shown to bind several other transition metals *in vitro* (Brazier et al., 2008; Jackson et al., 2001).

Several investigations using recombinant PrP and PrP peptides have shown that manganese can bind to PrP (Brown et al., 2000; Gaggelli et al., 2005; Jackson et al., 2001; Treiber et al., 2007). Perhaps the most comprehensive study to date, by Brazier *et al*, has demonstrated that one manganese atom can bind to the octarepeat region, however the highest affinity binding occurs at the fifth site (amino acids 95,110). The affinity of manganese to be PrP has been shown to be comparable to several other manganese binding proteins, suggesting that manganese binding to PrP is physiologically relevant (Brazier et al., 2008).

MnPrP has been shown to have an increased β-sheet content (Brown et al., 2000) and further work has shed more light on the structural changes that occur when the prion protein binds manganese. Studies using near infrared spectroscopy have shown that while bound to copper PrP is stable in aqueous solution, manganese bound PrP

is not protected from interactions with water and forms fibrils in an aqueous solution (Tsenkova et al., 2004). Work using Raman Spectroscopy showed that the initial binding of manganese to PrP resulted in an increase in α-helical structure (Zhu et al., 2008). As this is in contrast to work on aged protein that showed an increase in β-sheet structure (Brown et al., 2000), this suggests that ageing of manganese bound PrP is required to produce a PrP^{sc}-like structure. This correlates with our earlier work showing that aging is required to increase toxicity of MnPrP.

Our model may also have some *in vivo* relevance; there is increasing evidence to suggest manganese may be involved in prion disease. It has been demonstrated that manganese levels are raised in both blood and the CNS of patients suffering from prion disease (Hesketh et al., 2008) as well as in cattle infected with BSE and sheep infected with scrapie (Hesketh et al., 2007). Manganese is also associated with PrP purified from infected brains and cultured cells (Brown et al., 2000; Thackray et al., 2002; Wong et al., 2001b).

The concentration of MnPrP required to induce cell death in both N2a and F21 cells was relatively high. However other studies using full-length recombinant PrP in a PrP^{sc} like form have used concentrations of about 10 μg/ml to induce apoptosis in SH-SY5Y cells (Novitskaya et al., 2006), this is in the same concentration range required to induce toxicity in our model although much higher doses were required to produce a complete toxicity curve.

Initial studies have demonstrated that MnPrP is a valid model of prion disease, however the mechanisms leading to cell death remain unclear. There are several studies that investigate manganese binding to the prion protein and its effect on structure (Brazier et al., 2008; Treiber et al., 2007; Zhu et al., 2008). However as yet, studies have not investigated the regions of PrP that are involved in the structural conversion that occurs when PrP binds manganese and the regions required for metal binding, leading to cellular toxicity. This work would help us to understand the regions important in MnPrP toxicity.

3.5.2 Manganese Toxicity

As manganese is highly toxic to neurones it could be hypothesised that the cell death observed in our model is due to manganese toxicity. Our investigations have clearly shown that the levels of manganese added to the cultures, whilst approaching toxicity at the highest dose, do not cause the massive cell death observed when the cells are treated with MnPrP. Chronic manganese poisoning leads to symptoms resembling idiopathic parkinsonism (Calne et al., 1994). At the cellular level it has been demonstrated that manganese induces apoptotic cell death in PC12 cells, a rat pheochromocytoma cell line, however this cell death is not caspase dependant (Roth et al., 2000) unlike the cell death observed in our model which is due to caspase 3 activation. Therefore we can exclude manganese toxicity as the cause of cell death in our model.

3.5.3 Metal Imbalance and Other Neurodegenerative Diseases.

As well as studies suggesting that aberrant metal binding may be involved in prion disease there is increasing evidence that metal imbalance maybe relevant in other neurodegenerative diseases. In Alzheimer's disease (AD) high levels of copper, zinc, and iron are found in and around amyloid plaques (Lovell et al., 1998). Previous studies have shown that amyloid beta (Aβ), the main constituent of amyloid plaques in the brains of AD patients, can bind copper and zinc (Talmard et al., 2007; Tougu et al., 2008). Copper binding to Aβ 25-35 has been shown to promote conversion to a more β-sheet structured peptide that is more toxic *in vitro* (Giuffrida et al., 2007). Interestingly it has also been suggested that zinc binding to Aβ can cause aggregation (Tougu et al., 2008), however the addition zinc to cells in culture can prevent Aβ induced apoptosis by preventing Aβ aggregation (Cardoso et al., 2005).

Alpha-synuclein is a protein associated with Parkinson’s disease. It has been shown to bind copper (Rasia et al., 2005) and iron (Binolfi et al., 2006; Golts et al., 2002). Both copper (Rasia et al., 2005) and iron have been shown to increase alpha-synuclein aggregation (Ostrerova-Golts et al., 2000). However there is no direct evidence that metal binding increases the toxicity of alpha-synuclein.

This suggests that out model using aberrant metal binding may also be useful to model other neurodegenerative diseases as there is increasing evidence that metals play a role in neurodegenerative diseases.

3.5.4 Chronic Scrapie Infection

Cell lines chronically infected with scrapie have been widely used as tools for prion research, especially for investigating possible compounds useful for prion therapeutics (Pankiewicz et al., 2006; Webb et al., 2007). Certain cell lines seem to be able to maintain scrapie infection without any detrimental effect to the cell line. One such cell line is the SMB cell line, which is infected with the Chandler strain of scrapie (Clarke and Haig, 1970).

We investigated the toxicity of MnPrP on SMB cells, interestingly we found that scrapie infected cells were susceptible to MnPrP toxicity and cell death was apoptotic. Further investigation revealed that scrapie infected cells were also susceptible to the toxic peptide PrP 106-126, as were F21 cells. We hypothesised that scrapie infected cells were vulnerable to MnPrP toxicity as they are not resistant to PrP toxicity, as is often assumed, but that the levels of PrP^{sc} produced by the scrapie infected cells are low enough to be tolerated by the cells. This fits with previous work which has demonstrated that whilst cells can be infected with scrapie the infection rate can be as low as 1% in N2a cells (Race et al., 1987) rising to 13% infection in N2a cells over expressing PrP (Nishida et al., 2000). It also fits with observations in our laboratory that the availability of more PrP^{c}, which can be converted to PrP^{sc} is detrimental to the cells. As over-expression of PrP by stable transfection is not possible as cells lose expression after a few passages either due to slowing of division or death of transfected cells.

In order to investigate this hypothesis we treated both scrapie infected and F21 cells with PK digested lysate obtained from scrapie infected cells. This was shown not to be toxic to the cells, leading us to the conclusion that our hypothesis was correct and scrapie infected cells express levels of PrP^{sc} that are low enough to be tolerated by the cells. However it may be useful, in future work, to confirm our hypothesis that

SMB cells have a low level of infection by investigating the percentage of cells that are producing PrP^{sc}.

This chapter has shown that MnPrP is a suitable model of PrP^{sc}. It demonstrates many of the properties of PrP^{sc}, including an increase in β-sheet structure (Brown et al., 2000) and increased protease resistance. As it the full-length prion protein it is relevant *in vivo* unlike some peptides. There is also increasing evidence that manganese binding to the prion protein could occur *in vivo* (Brazier et al., 2008), suggesting that this model could represent a real disease mechanism.

Work treating SMB cells with MnPrP has demonstrated that cell death in infected cells is as a result of apoptosis. It would be useful to confirm that the cell death observed in F21 cells is due to apoptosis. Further investigation into the cell signalling mechanism that leads to apoptosis may provide possible therapeutic targets and work will also investigate the regions of MnPrP required for toxicity, this will help us to understand the regions important in the conversion of the prion protein into a toxic form.

4. PrP Toxicity

Previous studies have demonstrated that once PrP is converted from the normal cellular form to PrP^{sc} it is toxic to neurones. The toxicity of PrP^{sc} has been replicated using a variety of models, including peptides (Chabry et al., 2003; Forloni et al., 1993), recombinant protéine (Novitskaya et al., 2006) and expression of toxic mutants of PrP (Norstrom et al., 2007). The toxicity of the protein appears to be tied the increase in β-sheet that occurs when PrP^{c} is converted to PrP^{sc}. *In vitro* the increase in β-sheet content observed in PrP^{sc} has been replicated using a variety of methods, including the addition of chemicals such as SDS (Xiong et al., 2001) or by the addition of metals, including manganese (Kim et al., 2005).

PrP is able to bind manganese at two sites in the protein, these binding sites are in the octarepeat region (Jackson et al., 2001) and the so called fifth site (Brazier et al., 2008). The fifth binding site consists of two histidines located at amino acids 95 and 110 that have been shown to bind copper. A Met at residue 121 is also involved in this binding (Gaggelli et al., 2005). Manganese also binds at this site but in a differing manner, as it does not displace copper (Brazier et al., 2008). The octarepeat region also contains four potential binding sites located in the region stretching over amino acids 51-90. This region has been shown to bind manganese at low affinity. The octarepeat region also been hypothesised to be important in the disease, partly because an increase in the number of octarepeat regions from 5 up to 14 is one of the mutations observed in fatal familial insomnia, an inherited form of the disease (Collinge, 1997; Krasemann et al., 1995). Other investigations have also shown that mice expressing prion protein devoid of the octarepeat region have longer disease incubation times (Flechsig et al., 2000) leading to the belief that that this region has a role to play in the pathogenesis of the disease, although the octarepeat region is not thought be to be required for infection (Flechsig et al., 2000) suggesting a more complex picture.

The C-terminus region has been shown to contain the only known histidine mutation involved in inherited forms of the disease, at amino acid 187 (Cervenakova et al., 1999) as well as several other mutations involved in GSS. Cleaved fragments of the

C-Terminus have also been found in the brains of people suffering with CJD, and these fragments have been shown to be PK resistant (Zanuy et al., 2004). The C-Terminus consists of three α-helices, one of which was been shown to be toxic to cells in the presence of copper, although why copper was required for toxicity was not clear (Thompson et al., 2000). Whilst there are three histidines in the C-Terminus it is not clear whether these can bind metals, the histidine at 140, has been shown not bind copper however the results for the other histidines remained much less clear due to aggregation of the peptides used (Cereghetti et al., 2001). Further work, using peptides has shown that histidine 187 may be able bind metals (Brown et al., 2004).

Whilst there has been intensive study of the prion protein and the regions involved in toxicity the regions required for toxicity are still unclear. This study intends to investigate the effect of manganese on prion protein toxicity, as it has been hypothesised that manganese binding to the prion protein may be involved in the pathogenesis of prion disease. Any regions involved in the toxicity of MnPrP may also play a role in its conversion to a more β-sheet like protein. We have investigated a variety of mutants including mutants missing metal binding regions, the hydrophobic region and both N-terminal and C-terminal mutants. We have found that removal of all the metal binding regions significantly reduces the toxicity of MnPrP. We have also shown the importance of the presence of the octarepeat region, for the stability of the prion protein.

4.1 Toxicity of prion protein mutants

4.1.1 Metal Binding regions

Initially we investigated whether the mutants we were investigating were intrinsically toxic, that is toxic even without manganese bound. We investigated the null mutant, which has the histidines at sites 66, 68, 76, 84, 96 and 111 mutated into alanines, these are all the histidines currently thought to be involved in metal binding in the prion protein. Previous work has demonstrated that the null mutant does not bind copper using Raman (Zhu et al., 2008).

There are no histidines present in the octarepeat region, so the protein does not bind to a copper loaded IMAC column, therefore the protein was purified by the wash

method, as described in the materials and methods. F21 cells were treated for 48h with varying concentrations of null MnPrP or null apoPrP for 48h and viability assessed using an MTS assay.

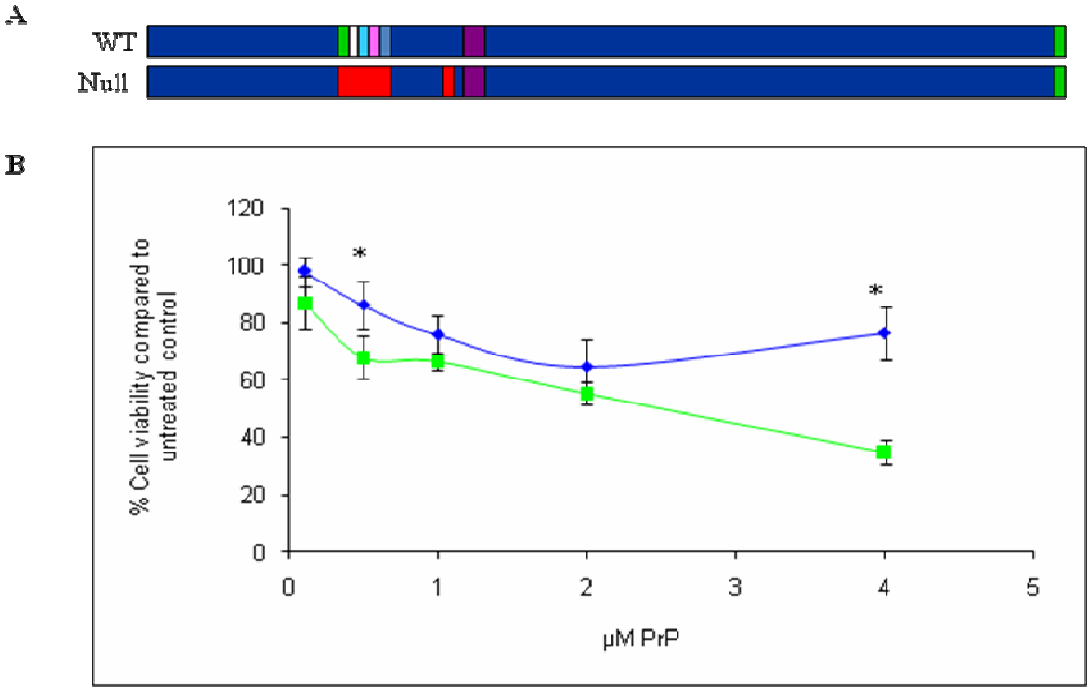


Figure 4.1: Toxicity of Null PrP. A: A schematic diagram showing the mutations in the null prion protein, the red bars represent the mutation of histidines to alanines in the regions thought to bind metals the top diagram shows the WT protein. B: F21 cells were treated for 48h with either recombinant Null apoPrP (blue line) or Null MnPrP (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

There was not a dose dependant difference between the null apoPrP and the manganese bound null PrP (figure 4.1) although there was a significant difference in toxicity at two doses, most notably the highest dose. The reason for this difference is unclear and requires further investigation As previous studies have shown that null does not bind copper (Zhu et al., 2008) and a recent study has also shown that the null mutant does not bind manganese (Brazier et al., 2008) it was expected that there would be no difference in toxicity whether refolded in the presence of manganese or not.

Having investigated the toxicity of a mutant unable to bind manganese we investigated several mutants that had mutations in the octarepeat region. Initially we investigated the effect of the removal of the octarepeat region on toxicity using

deletion mutants. We first used a mutant that had had the entire octarepeat region removed, PrP Δ51-89. This protein was purified by nickel IMAC via a his-tag and F21 cells were treated for 48h with varying concentrations of MnPrP Δ51-89 or apoPrP Δ51-89 for 48h and viability assessed using an MTS assay. PrP Δ51-89 was found to be significantly less toxic than manganese bound Δ51-89 (figure 4.2b). This suggests that manganese binding does have an effect on the toxicity of PrP that is missing the octarepeat region, suggesting that manganese binding to the fifth site, which is the only site available to bind to, is able to modulate toxicity.

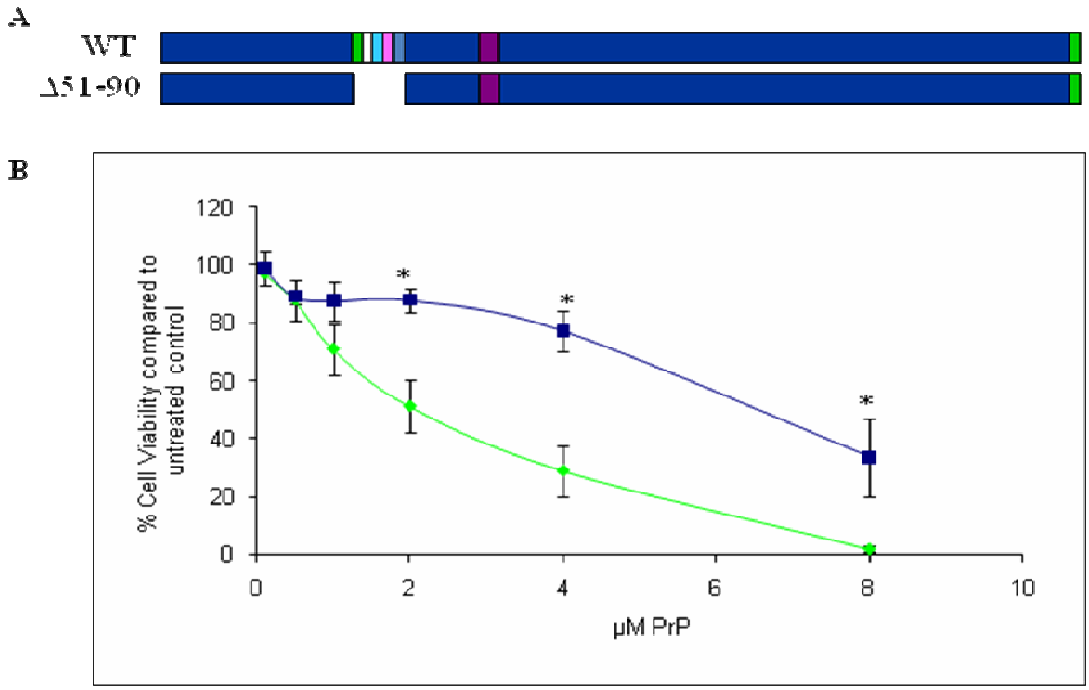


Figure 4.2: Toxicity of PrP Δ51-89. A: A schematic diagram showing the mutations in the Δ51-89, the removal of octarepeat can be seen the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP Δ51-89 (blue line) or MnPrP Δ51-89 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

We investigated whether partial removal of the octarepeat region had any effect on toxicity, to investigate this we used a mutant (Δ67-89) that has had all but the first repeat removed leaving one copper binding site in the octarepeat region as well as the fifth site. This protein was purified by nickel IMAC via a his-tag, F21 cells were then treated with varying concentrations of MnPrP Δ67-89 or apoPrP Δ67-90 for 48h and toxicity was assessed using an MTS assay. The Δ67-90 apoPrP was significantly less toxic than the metal bound PrP (figure 4.3).

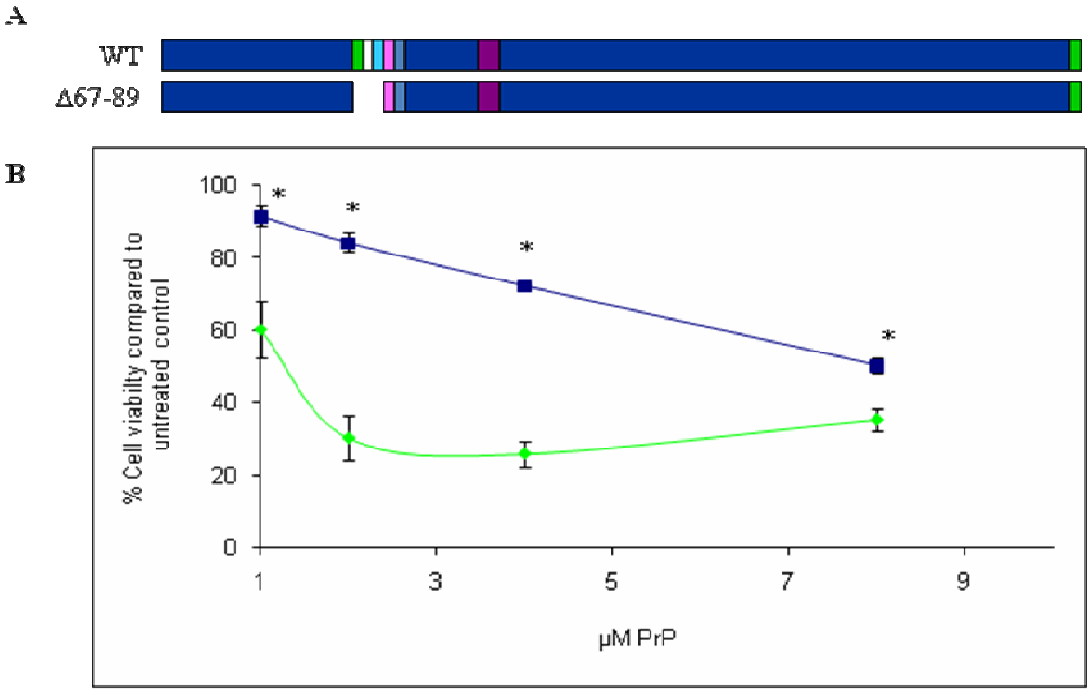


Figure 4.3: Toxicity of PrP Δ67-89. A: A schematic diagram showing the mutations in the Δ67-89, the partial removal of octarepeat can be seen the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP Δ67-89 (blue line) or MnPrP Δ67-89 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 3 repeats.

The removal of such a large part of the protein may lead to structural changes in the prion protein. To further investigate the effect of metal binding on the toxicity of the prion protein we choose to use a series of mutants that had had the histidines involved in metal binding mutated to alanines. This allowed us to investigate the effect of metal binding without the possibility of dramatically changing the structure of the protein.

We investigated the effect of mutating the histidines in the octarepeat region into alanines to prevent binding. This left only the fifth site available for binding. As with the null mutant this protein was purified by the wash method and F21 cells were treated as previously described, with MnPrP Fifth site only or apoPrP Fifth site only. As with the previous two mutants we observed an increase in toxicity when manganese was bound to the protein suggesting manganese binding to the fifth site increases toxicity (figure 4.4), however it is noticeable that the addition of

manganese appears to increase toxicity more than in the deletion mutants, as even at the highest dose used apoPrP fifth only shows very little toxicity.

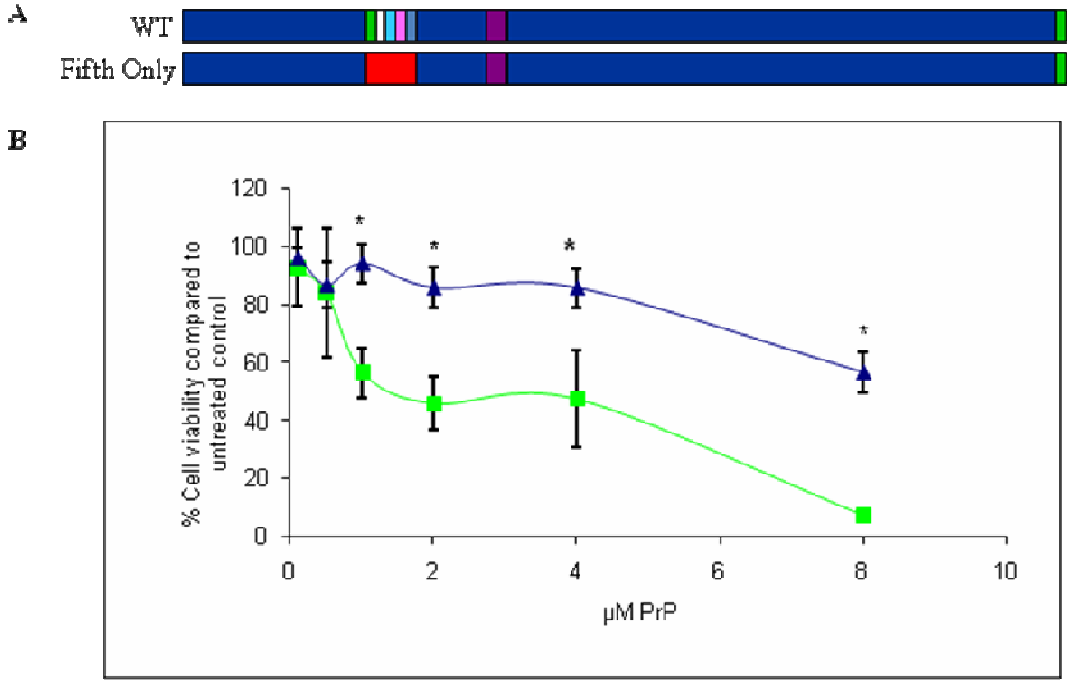


Figure 4.4: Toxicity of PrP Fifth only. A: A schematic diagram showing the mutations in the fifth only prion protein, the red bar represents the mutation of histidines to alanines in the octarepeat region thought to bind metals, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either recombinant Fifth only apoPrP (blue line) or Fifth Only MnPrP (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

We also investigated the effect of the loss of the fifth site on toxicity. A mutant with the histidines at 95 and 110 mutated into alanines was purified using the wash method. F21 cells were treated for 48h with either no fifth apoPrP or no fifth MnPrP and viability was assessed using an MTS assay. The removal of the fifth site did not lead to a loss of toxicity, manganese bound PrP no fifth site was significantly more toxic then apoPrP no fifth site, suggesting that binding to the octarepeat region may have some effect on toxicity (figure 4.5).

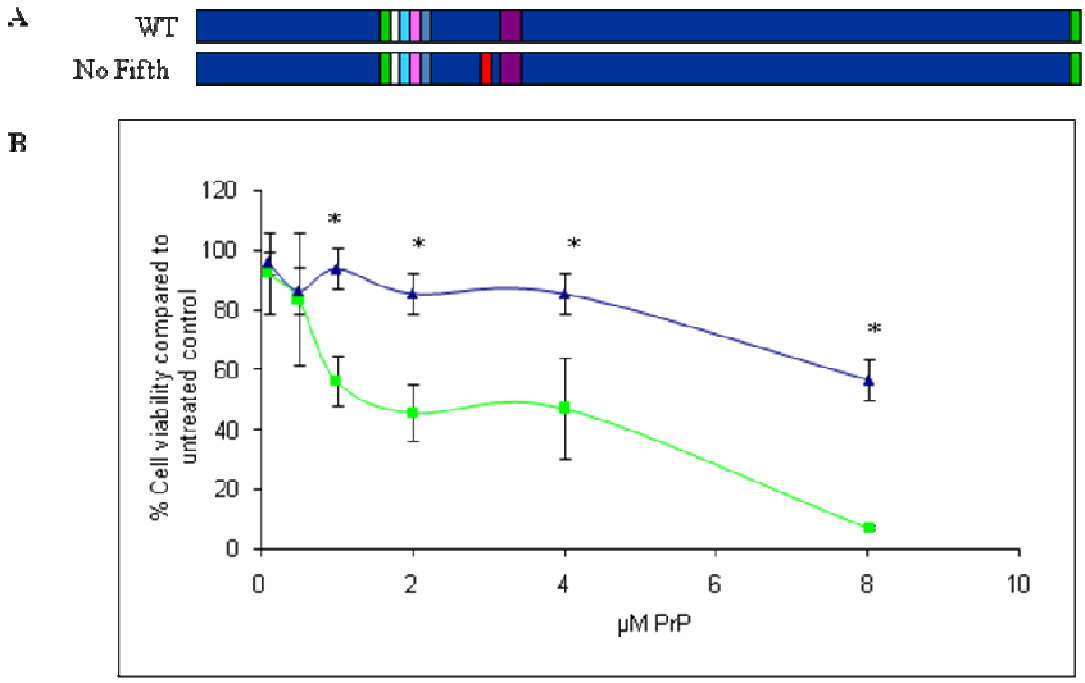


Figure 4.5: Toxicity of PrP No Fifth. A: A schematic diagram showing the mutations in the no fifth prion protein, the red bar represents the mutation of histidines to alanines at the fifth site thought to bind metals, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP no fifth (blue line) or MnPrP no fifth (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

4.1.2 N-Terminal Region.

The N-terminal region of the prion protein is defined as amino acids 23-112. We have investigated two mutants that cover the N-terminal region one spanning amino acids 23-112 and the other spanning amino acids 23-171. The N-terminal region of PrP is thought be unstructured (Donne et al., 1997), although it has been reported that metal binding may confer more structure on the region (Jones et al., 2004).

Initially we investigated PrP 23-112, which is missing amino acids 112-231. F21 cells were treated for 48h with varying concentrations of MnPrP 23-112 or apoPrP 23-112 and viability was assessed using an MTS assay. MnPrP 23-112 showed an increase in toxicity when bound to manganese (figure 4.6), as this region still has all the metal binding histidines available to bind it is perhaps unsurprising that it is more toxic when bound to manganese.

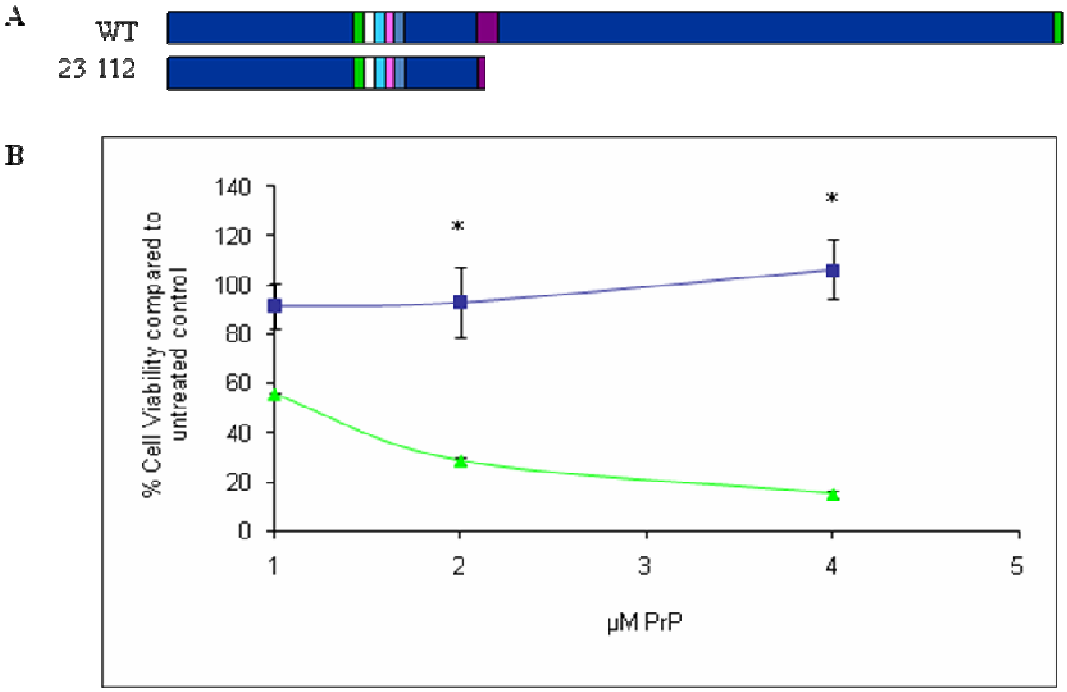


Figure 4.6: Toxicity of PrP 23-112. A: A schematic diagram showing the 23-112 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP 23-112 (blue line) or MnPrP 23-112 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

We also investigated a bigger fragment of the N-terminal end of the protein, amino acids 23-171, which is missing residues 172-231. As before cells were treated for 48h with MnPrP 23-171 or apoPrP 23-171 and viability was assessed using an MTS assay. This fragment was shown to be toxic when bound to manganese, however it was also shown to be toxic without metal bound to it, leading us to believe that this is an intrinsically toxic fragment of the prion protein (figure 4.7).

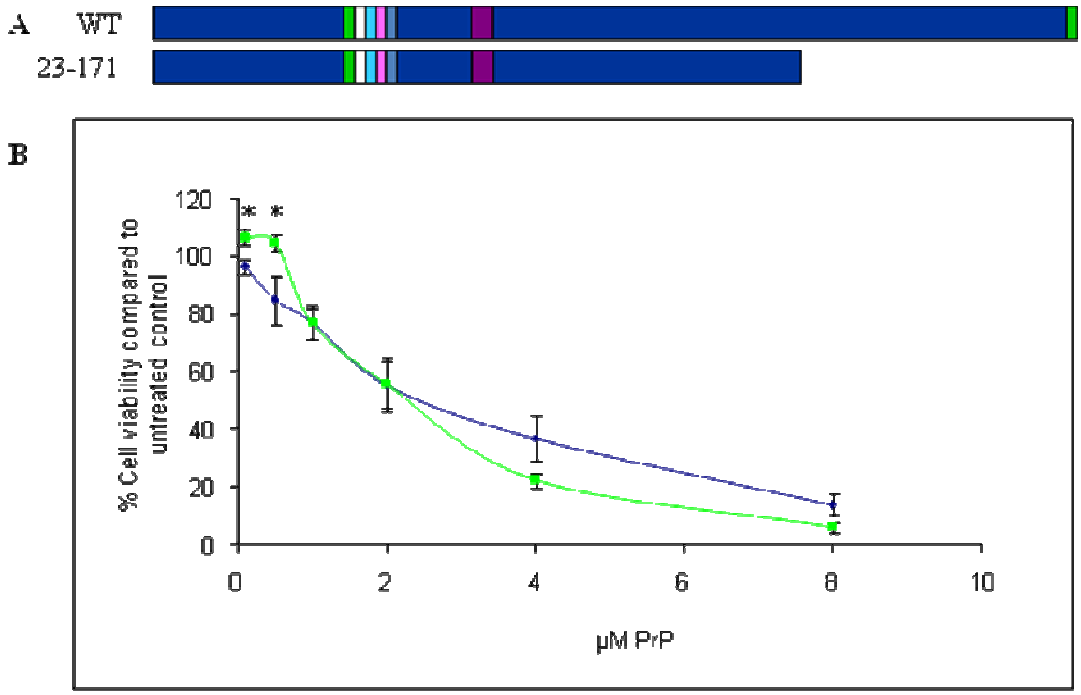


Figure 4.7: Toxicity of PrP 23-171. A: A schematic diagram showing the 23-171 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP 23-171 (blue line) or MnPrP 23-171 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

4.1.3 Palindromic Region.

The palindromic region of the prion protein consists of 8 amino acids extending from amino acid 112 to 119. The sequence (AGAAAAGA) is also known as the hydrophobic core. Work has using the 106-126 peptide demonstrated the replacing hydrophobic amino acids in the palindromic region with hydrophilic amino acids resulted in a loss of toxicity and marked decrease in β-sheet structure (Jobling et al., 1999). The absence of the palindromic region prevents the conversion to PrP^{sc} (Norstrom and Mastrianni, 2005).

Cell were treated for 48h with varying concentrations of MnPrP Δ112-119 or apoPrP Δ112-119. Interestingly, although the protein bound to metal is significantly less toxic than the recombinant protein this difference in toxicity is strikingly less than seen in other mutants (figure 4.8), this may suggest that the fragment is, at intrinsically toxic although metal binding does confer a very slight increase in toxicity.

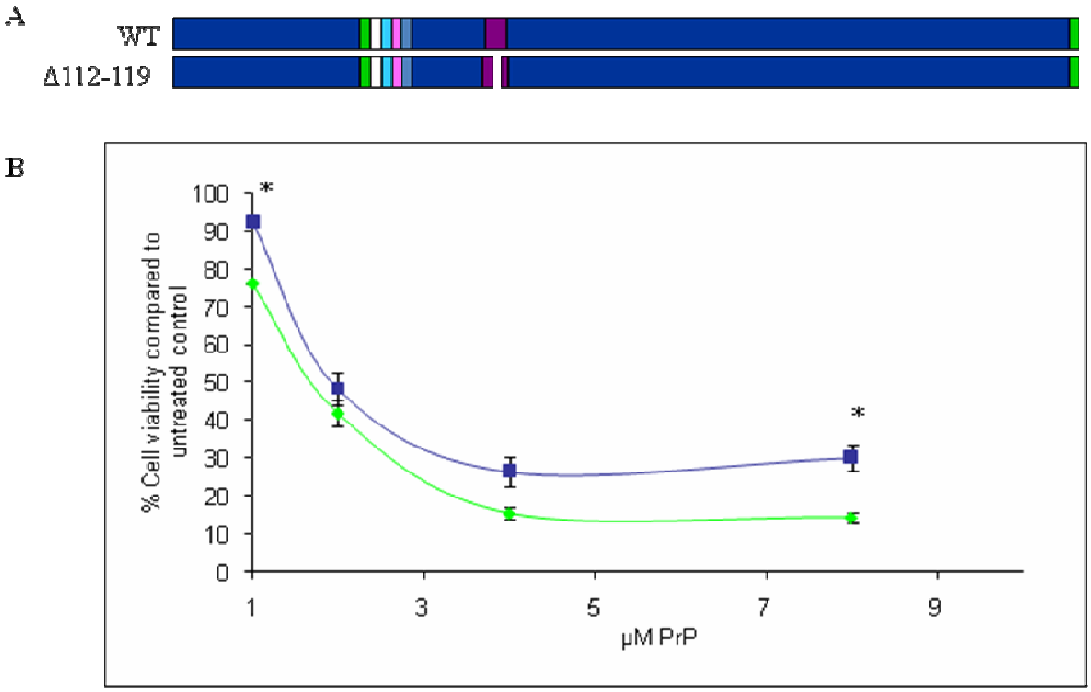


Figure 4.8: Toxicity of PrP Δ112-119. A: A schematic diagram showing the Δ112-119 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP Δ112-119 (blue line) or MnPrP Δ112-119 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

4.1.4 112-231

The C-terminus region of the prion protein comprises amino acids 112-231 and is highly structured. In the normal prion the structure consists of 3 α-helices and two short β-sheet strands.

Our results have shown that the C-terminus is not intrinsically toxic to the cells (figure 4.9), in fact this protein, when not bound to metal, appears not to be toxic to cells at any concentration. This is in sharp contrast to most other mutants which show toxicity at high doses. Previous work has shown that cellular PrP expression protects cells from the toxicity of the C-terminal fragment of PrP (Daniels et al., 2001).

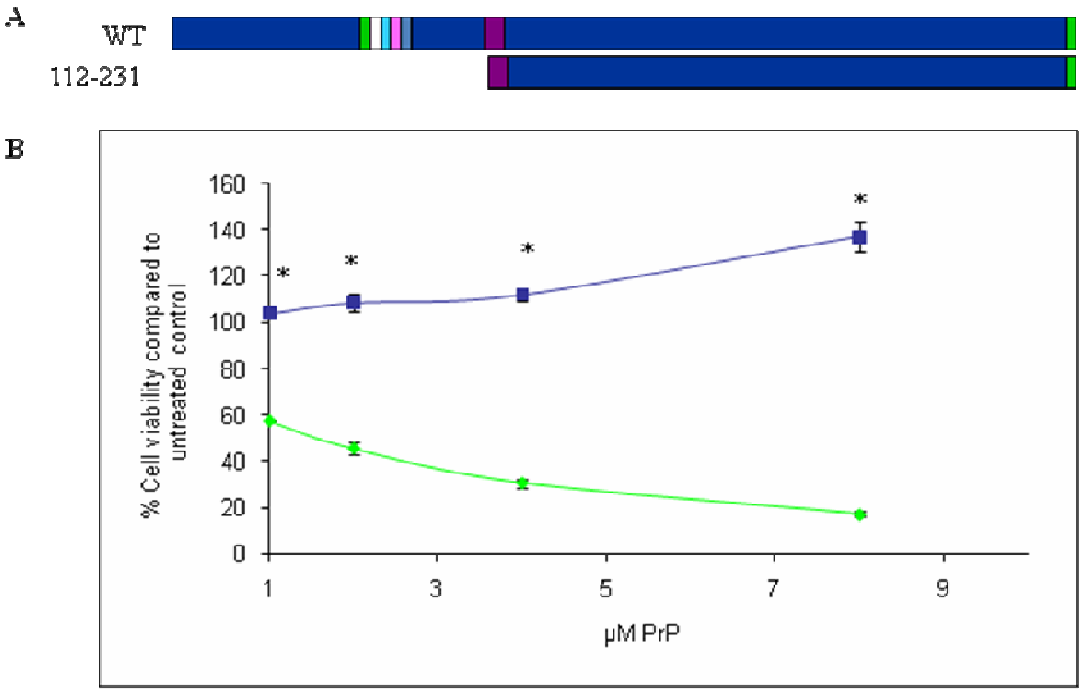


Figure 4.9: Toxicity of PrP 112-231. A: A schematic diagram showing the 112-231 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP 112-231 (blue line) or MnPrP 112-231 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

A mutant encompassing more of the protein, 45-231 was also investigated, this protein contains all of the metal binding regions and is missing just the first 22 amino acids. This protein was not found to be intrinsically toxic (figure 4.10) however it was toxic once bound to manganese.

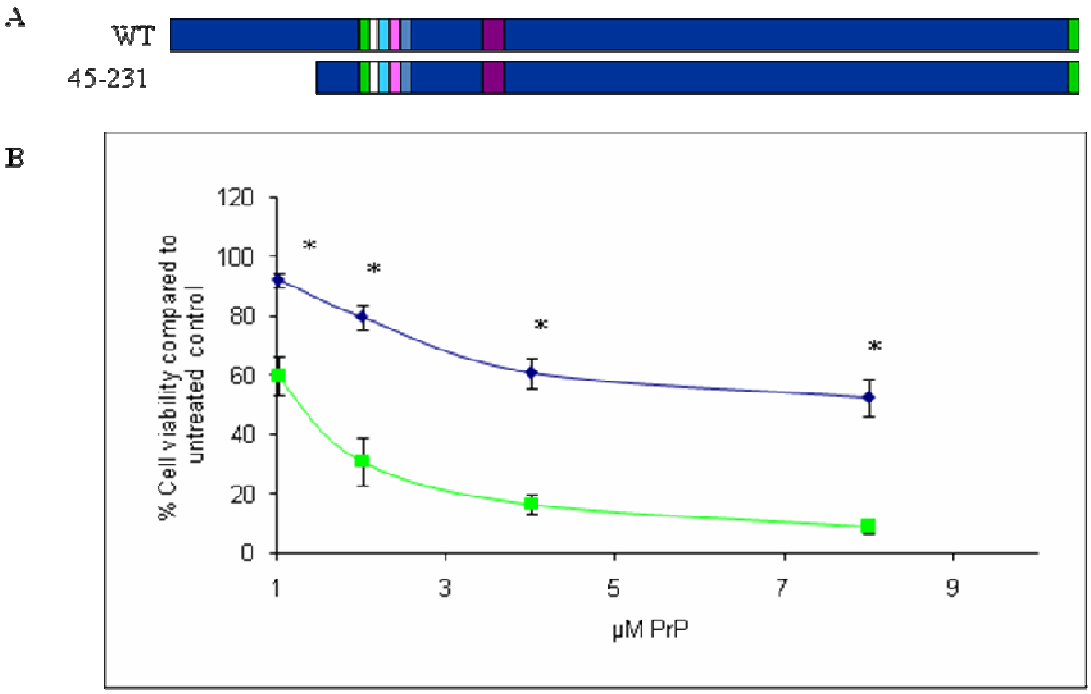


Figure 4.10: Toxicity of PrP 45-231. A: A schematic diagram showing the 45-231 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP 45-231 (blue line) or MnPrP 45-231 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

Finally we also investigated the toxicity of Δ135-150 and Δ35-45. Neither of these deletions has had any reported effect on the proteins ability to bind metals and neither was intrinsically toxic to the cells (figures 4.11 & 4.12). However manganese binding significantly increased the toxicity of protein.

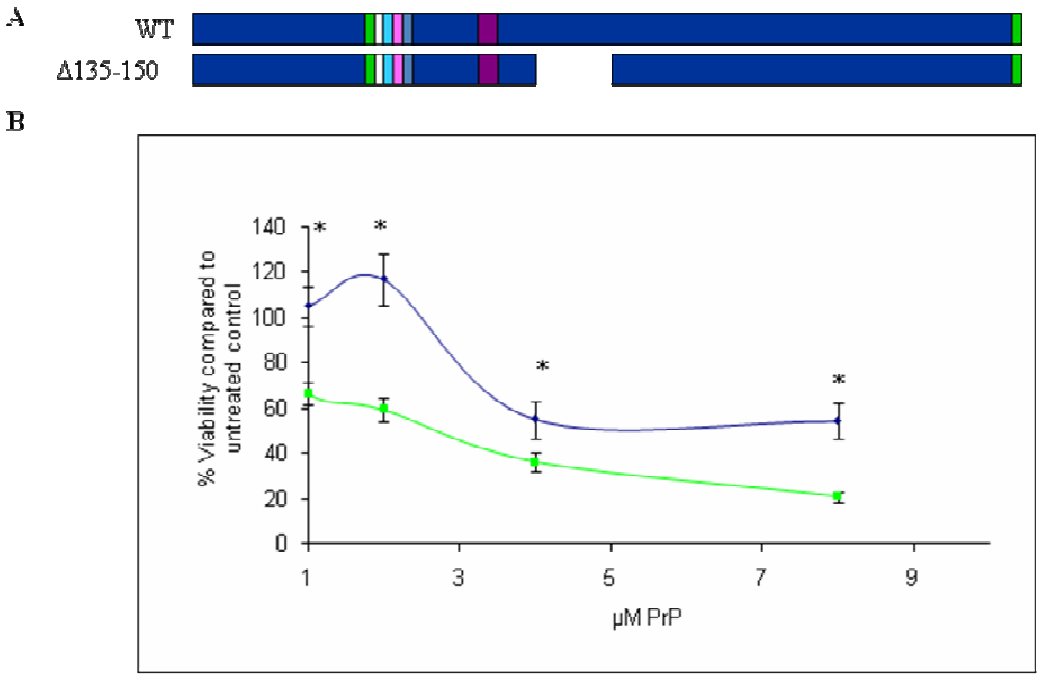


Figure 4.11: Toxicity of PrP Δ135-150. A: A schematic diagram showing the Δ135-150 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP Δ135-150 (blue line) or MnPrP Δ135-150 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

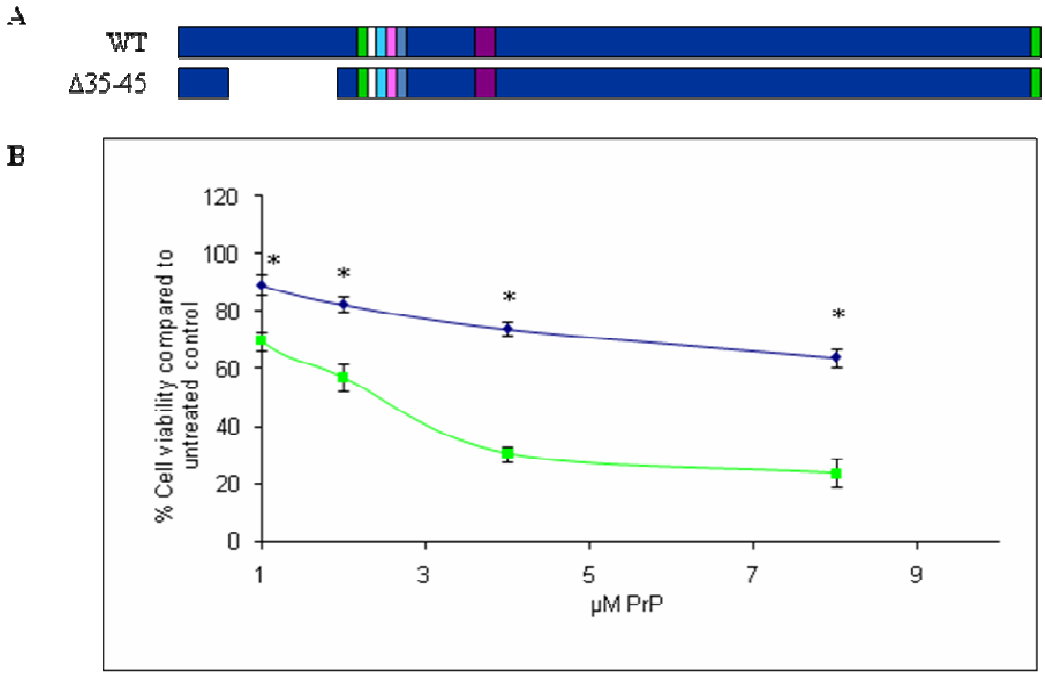


Figure 4.12: Toxicity of PrP Δ35-45. A: A schematic diagram showing the Δ35-45 prion protein, the top diagram shows the WT protein. B: F21 cells were treated for 48h with either apoPrP Δ35-45 (blue line) or MnPrP Δ35-45 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

4.2 Comparison of MnPrP WT toxicity to the mutants.

Having established the toxicity of each mutant when bound to manganese we wanted to investigate which regions of the protein were responsible for the increased toxicity of MnPrP when compared to apoPrP. F21 cells were treated for 48h with varying concentrations of WT PrP or mutant PrP bound to manganese, and an ED_{50} for each mutant was established as well as a dose response curve. The results are summarised in a table on page 92.

We used both the wash method and his-tag to purify PrP, depending on the mutant used. The his-tagged protein was significantly less toxic than the protein purified by the wash method. The reasons for this are unclear, it may be due to slight differences in the protein produced by each method as they are very different methods for producing protein. However due to these differences in toxicity the mutants were compared back to the relevant wild type protein results.

4.2.1 Metal binding Regions

The null mutant was significantly less toxic than the wild type protein with an ED_{50} of 3.28µM compared to WT, which had an ED_{50} of 1.96µM. It is not entirely surprising that the null mutant is less toxic than WT given that the null mutant is not thought to bind manganese. The graph shows that the difference was not as great at the highest dose (figure 4.13b) but this point may not be as relevant as the two lower doses, as this is approaching the concentration where the levels of manganese may become toxic to the cells and the large volumes of protein added to the cells may affect the cell culture media buffering. This result suggests that removal of the sites currently thought to bind manganese can reduce but not obliterate the toxicity of the prion protein.

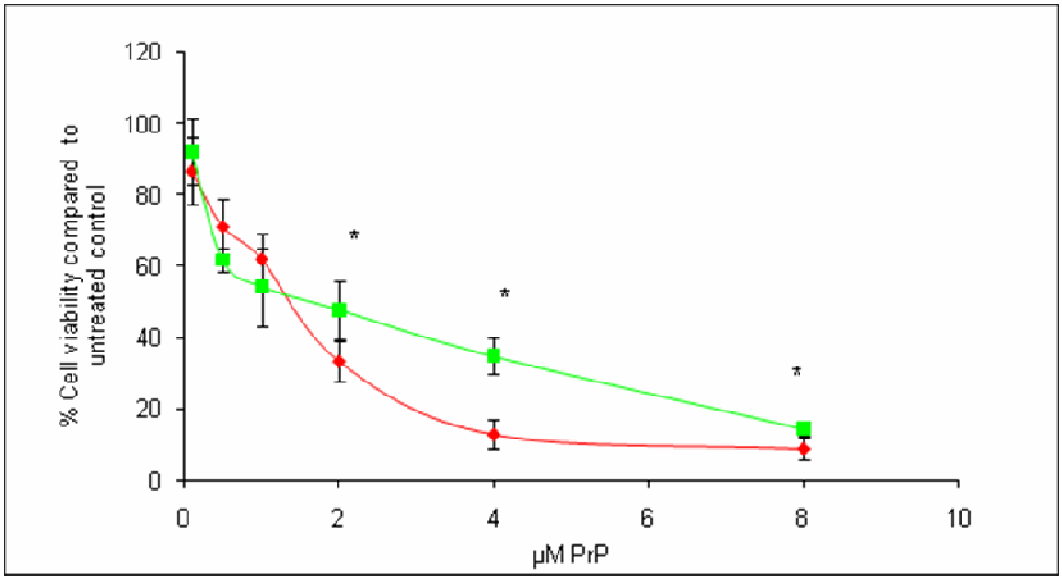


Figure 4.13: Toxicity of Null MnPrP compared to WT MnPrP. A: F21 cells were treated for 48h with either WT MnPrP (red line) or Null MnPrP (green line) or WT MnPrP (column purified) (yellow line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

We observed increases in toxicity in both the mutants that had had the octarepeat removed, even when the octarepeat was only partially removed. Full removal of the octarepeat region gave an ED_{50} value of 2.17µM and partial removal gave an ED_{50} of 2.47µM these are both much lower than the wild type PrP value of 3.94µM. Dose response curves for both mutants also showed a significant increase toxicity when compared to the WT (figure 4.14).

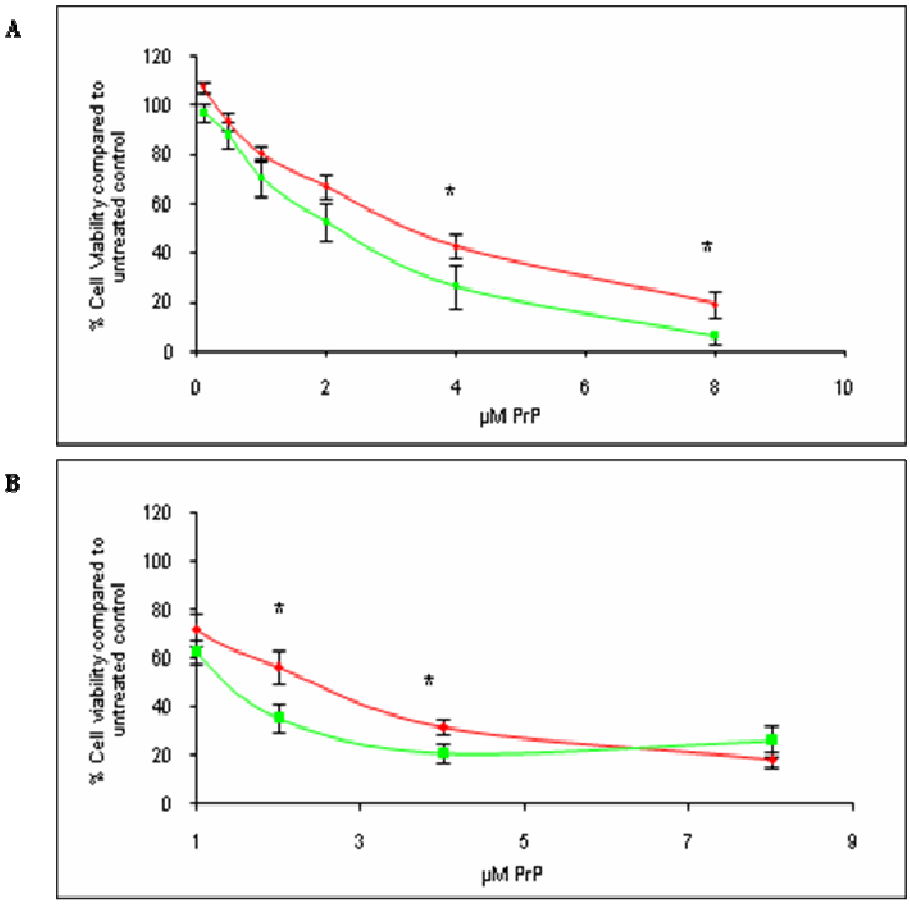


Figure 4.14: Toxicity of Δ51-89 and Δ67-89 MnPrP compared to WT MnPrP. A: F21 cells were treated for 48h with either WT MnPrP (red line) or Δ51-89 MnPrP (green line). B: F21 cells were treated for 48h with either WT MnPrP (red line) or Δ67-89 MnPrP (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

As deletion of the octarepeat increased the toxicity of MnPrP we investigated the toxicity of a mutant that had all the histidines in the octarepeat region replaced with alanines (fifth site only mutant). This allowed us to assess whether the increase in toxicity was due to the metal binding sites or changes in structure induced by the removal of the octarepeat. We confirmed that the increase in toxicity was also observed in the fifth site only mutant (figure 4.15) indicating that when both the fifth site and the octarepeat have metal bound this may give a slight protective effect compared to the fifth is on its own.

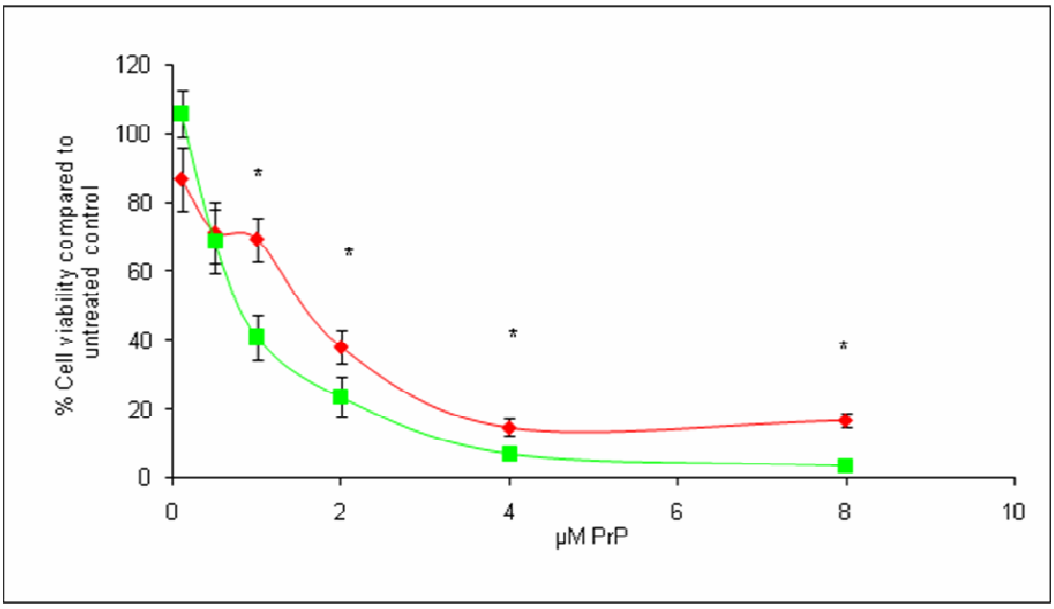


Figure 4.15: Toxicity of fifth site only compared to WT MnPrP. A: F21 cells were treated for 48h with either WT MnPrP (red line) or fifth site only (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

We also investigated the effect of the removal of the fifth site leaving just the octarepeat region. Interestingly it was found that this mutant showed no significant difference in toxicity when compared to WT MnPrP and had a very similar ED_{50} value to the WT value. Taken with the results using the null and fifth site only mutants this suggest that the fifth site bound to manganese can increase the toxicity of the prion protein and that the octarepeat region is not able to modulate the toxicity of the protein.

4.2.2 N-Terminal Region.

We investigated the effect of removal of the structured C-terminus of PrP on MnPrP toxicity using the 23-112 mutant. The other mutant that covered the N-terminus was found to be intrinsically toxic so was suitable for investigating the role of metal binding in PrP toxicity. The N-terminus of PrP was found to be highly toxic with an ED_{50} value of 1.18µM, almost four times lower than the value for WT MnPrP and the dose response curve showed a significant increase in toxicity compared to the WT (figure 4.16). This is despite the presence of the all the metal binding sites, suggesting that the presence of the C-terminus might be important in the prevention of toxicity.

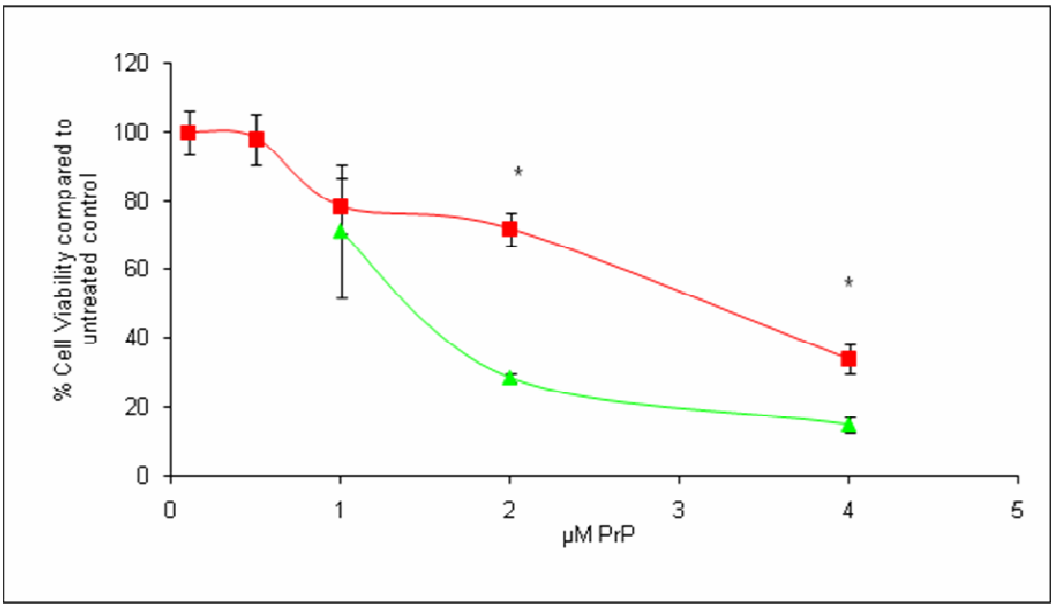


Figure 4.16: Toxicity of 23-112 MnPrP compared to WT MnPrP. A: F21 cells were treated for 48h with either WT MnPrP (red line) or N-terminal PrP (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

4.2.3 Hydrophobic Region

Cells were treated for 48h with varying concentrations of WT MnPrP or Δ112-119 MnPrP. Our work has shown that there is no difference in toxicity between WT and the Δ112-119 mutant suggesting that whilst this region is highly fibrillogenic and involved in the toxicity of the protein, the binding of manganese is not involved in this toxicity.

4.2.4 113-231

Our results have shown that the 113-231 shows a slight decrease in toxicity when compared to the WT MnPrP with an ED_{50} value of 4.77μM compared to the EC_{50} value of WT MnPrP which is 3.96μM. This is reflected in the dose response curve which shows a slight but significant decrease in toxicity (figure 4.17).

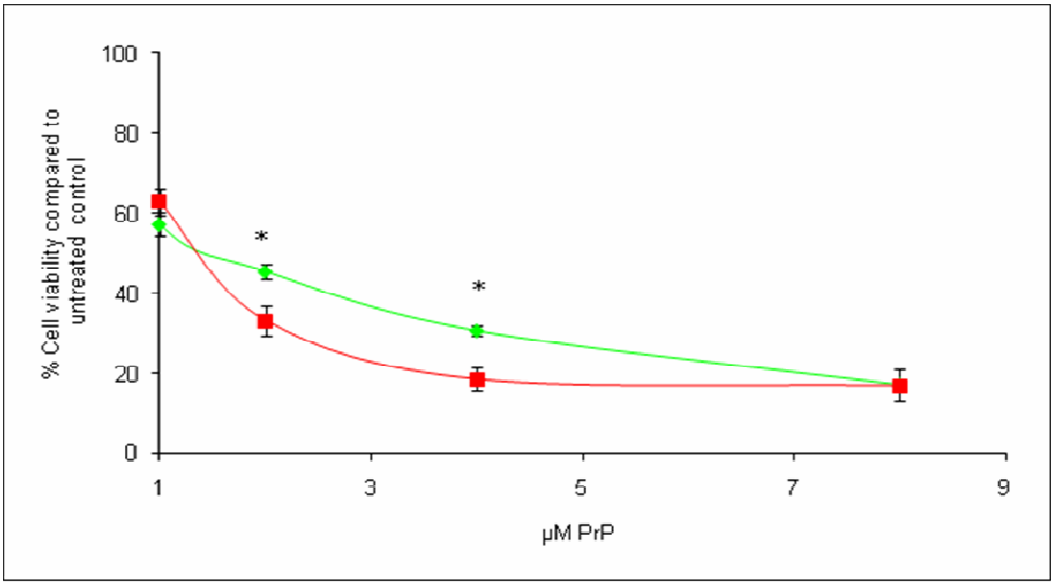


Figure 4.17: Toxicity of 113-231 MnPrP compared to WT MnPrP. A: F21 cells were treated for 48h with either WT MnPrP (red line) or 113-231 (green line). Cell viability was then assessed using an MTS assay error bars are SEM. Significant points were assessed using a one-way ANOVA $p<0.05$. Each point represents at least 4 repeats.

This decrease is toxicity may be due to the fact that no manganese is bound to the protein, although the difference in toxicity is not as great as observed in the Null mutant. Another mutant that has the N-terminus partially removed (45-231) showed no difference in toxicity compared to WT MnPrP. Interestingly the Δ35-45 mutant also had a much higher ED_{50} value than WT MnPrP, whilst the Δ135-150 mutant showed no difference.

A

Mutant	ED50 Compared to WT (μM)	Change in toxicity compared to control
WT His Overall	3.94	N/A
Δ112-119	3.16	No change
Δ51-90	2.17	Increase
Δ67-90	2.47	Increase
23-112	1.18	Increase
112-231	4.77	Decrease
Δ35-45	4.80	Decrease
Δ135-150	3.99	No change
45-231	3.71	No change
WT wash Overall	1.96	N/A
Fifth Only	0.73	Increase
Null	3.28	Decrease
No fifth	1.70	No change

B

Table 4.1: A: Shows the ED50 values for each manganese bound mutant compared to the relevant manganese bound WT protein. Differences in toxicity are given when there was a significant increase or decrease apparent on the chart. B: A schematic diagram highlighting some of the important regions of the prion protein.

4.3 Discussion

This study has highlighted the potential significance of manganese binding in the pathogenesis of prion disease. The results in the previous chapter showed that manganese binding increases the toxicity of the prion protein. In this chapter we have further investigated the role of manganese binding in prion toxicity. Our work has shown that if the histidines known to bind manganese are mutated the toxicity of the protein is significantly reduced suggesting binding of manganese is required for toxicity. We have also shown that removal of the octarepeat region increases the toxicity of MnPrP, suggesting that this region has a protective effect. In contrast removal of the fifth site has no effect on toxicity. However it might be that this is because manganese is bound to the octarepeat in this situation.

4.3.1 Metal Binding

As we had shown that manganese bound PrP was toxic to neuronal cells we investigated whether altering the metal binding ability of PrP affected the toxicity of the prion protein.

We assessed the role of metal binding sites and their effect on MnPrP mediated toxicity. The Null mutant, which has all the histidines known to bind metal replaced with alanines and is unable to bind manganese (Brazier et al., 2008). The Null mutant was found to be significantly less toxic than the WT protein at lower doses. This suggests that the binding of manganese to the WT prion protein leads to an increase in toxicity and confirms our earlier work that showed an increase in toxicity when manganese is bound to WT PrP.

Studies of a variety of neurodegenerative diseases have shown that metal ions can induce aggregation of proteins. Several studies have demonstrated that the prion protein aggregates in the presence of manganese (Giese et al., 2004; Kim et al., 2005; Treiber et al., 2006) and it has been suggested that manganese causes aggregation by creating interactions between PrP molecules rather than binding to the protein (Levin et al., 2005). Our data suggest that the increase in toxicity we observed with manganese is due to binding of the metal to specific regions of the

prion protein. There was no difference in toxicity between the recombinant Null PrP and the manganese bound Null PrP, which is probably due to the fact that there are no metal ions bound to either protein.

4.3.2 Octarepeat Region

Studies have shown that amino acids 23-90 are not required for prion propagation (Fischer et al., 1996; Muramoto et al., 1997; Muramoto et al., 1996) suggesting that the octarepeat region is not required for infectivity. Work using a fragment of PrP termed PrP106 or mini prion, which is 106 amino acids long and contains the deletions Δ23-88 and Δ141-176, has also suggested that the octarepeat region is not required for toxicity or infection. The mini prion is a soluble form of PrP that demonstrates several properties in common with PrP^{sc} (Muramoto et al., 1996), and work using recombinant PrP106 has show that it is was highly toxic to primary neuronal cultures (Bonetto et al., 2002).

However the octarepeat region may have a role in prion disease pathogenesis, as an increase in the number of octarepeat regions from 5 up to 14 is one of the mutations observed in fatal familial insomnia, an inherited form of prion disease (Collinge, 1997; Krasemann et al., 1995). A model using recombinant protein has shown that increasing numbers of octarepeat insertions result in increased aggregation of the prion protein (Yu et al., 2007). Mice expressing PrP with nine octarepeats accumulate an insoluble and weakly protease-resistant form of the mutant protein that is not infectious (Biasini et al., 2008).

In addition, work using mice expressing a prion protein mutant devoid of the octarepeat region develop prion disease with a longer incubation time (Flechsig et al., 2000) when challenged with infection, compared to normal mice. Further work using a cell culture model suggests that the octarepeat region of prion protein is required at an early stage for production of abnormal prion protein (Sakudo et al., 2008), however it may not be required later, PrP^{sc} is cleaved during trafficking and the 90-231 fragment is found in patients suffering from CJD (Chen et al., 1995).

There has been some debate on how many manganese atoms bind to the octarepeat region of the prion protein, some studies have not been able to detect any binding to

the octarepeat region (Garnett and Viles, 2003; Treiber et al., 2007) however more recent work suggests a single low affinity binding event in the octarepeat region (Brazier et al., 2008). PrP Δ51-90 has been shown to bind metals at the fifth site (Jones et al., 2004).

We found that both full and partial removal of the octarepeat region led to a significant increase in MnPrP toxicity. This result was confirmed using a mutant containing only the fifth site histidines and no octarepeat region histidines which was shown to be significantly more toxic than the wild type protein. Work investigating the effect of the deletion of the octarepeat region on toxicity has been limited, however there is evidence that deletion results in a loss of protective function (Haigh and Brown, 2006). In our model the increase in toxicity observed may be due to a loss of stability in the absence of metal binding in the octarepeat region. Studies have shown that a deletion mutant, PrP 90-231, which contains only the fifth binding site is unstable in the presence of copper and ascorbate due to the oxidation of the methionines and histidines in the fifth site region (Nadal et al., 2007). This oxidation can be partially prevented by the presence of the octarepeat region. If this oxidation were to take place in the fifth site only MnPrP mutant, in the presence of manganese, a potential effect could be an increase in aggregation, as oxidation of the prion protein has been shown to increase aggregation *in vitro* (Requena et al., 2001). Oxidation-induced aggregation may increase the toxicity of the protein, as fibrils and aggregates of recombinant PrP have been shown to be toxic to cells *in vitro* (Corsaro et al., 2006; Novitskaya et al., 2006). Additionally the loss of the octarepeat region may also result in a loss of stability, as proteins that do not contain the repeat region have been shown to be less stable under pressure (Cordeiro et al., 2005).

Several studies have shown that PrP is susceptible to cleavage in the presence of metals and H_2O_2 (McMahon et al., 2001). Cleavage of PrP^c occurs at around amino acids 111 and 112. PrP^{sc} cleavage occurs at round amino acid 90, although the normal protein can also be cleaved here (Mange et al., 2004). Further work has shown that PrP bound to manganese can be partially cleaved, in the presence of H_2O_2, at amino acid 90 and that this protein can cause WT PrP to aggregate. Interestingly PrP missing the octarepeat region was cleaved much more readily in the presence of copper and H_2O_2 resulting in almost complete cleavage at amino acid 90.

The increase in cleavage observed may result in an increased polymerisation of cellular PrP and therefore increased toxicity (Abdelraheim et al., 2006). This may also explain the increase in toxicity of MnPrP in the absence of the octarepeat.

As the presence of fifth site alone has an effect on toxicity it suggests that the binding pattern of our refolded protein is like that observed by Brazier *et al* with two manganese atoms binding to the prion protein one to the fifth site and one to the octarepeat region, rather than the earlier suggestion that four manganese atoms bind to the octarepeat region (Brown et al., 2000). This is also confirmed by the mass spectroscopy data, which showed two manganese atoms bind to the prion protein (Uppington and Brown, 2008)

4.3.3 Fifth Site

A small number of researchers have suggested an alternative hypothesis that the fifth site has a higher affinity for copper than the octarepeat region (Jones et al., 2005a; Treiber et al., 2007). Studies using peptide covering the region 91-115 have shown that this region predominately random coil when not bound to copper. Once bound to copper the peptide is more structured, showing an increase in β-sheet, accompanied by the loss of some of the irregular structure. This implies the fifth binding site may be important in determining structure (Jones et al., 2004). However as yet it is not clear whether manganese binding to the fifth alone site can also induce changes in structure.

Manganese has been shown to bind to the fifth site in a different manner to copper. Work using the 106-126 peptide has shown that the complex with Mn(II), involves the carbonyl oxygen's of Gly-124 and Leu-125, which are close to the metal ion, the metal ion interacts with His-110, through a hydrogen bond of metal-bound water (Gaggelli et al., 2005). However more recent studies have shown that binding is centralised round His-95 and manganese binding still possible even in the presence of copper (Brazier et al., 2008). The 5^{th} site is now thought to be the highest affinity site for manganese binding (Brazier et al., 2008). However, until now, is there is has been no direct work on the effect of metal binding to fifth site and its effect on toxicity. We observed no change in toxicity when the fifth site was mutated to

prevent metal binding at this site. This suggests that the weaker binding site in the octarepeat may be able to maintain the toxicity of the MnPrP without the presence of the fifth site but not increase it.

4.3.4 Hydrophobic Region

The region spanning amino acids 112 to 119 is highly conserved between species and has been shown to be the most fibrillogenic motif within the toxic peptide (Gasset et al., 1992).

The hydrophobic region is thought to be required for infectivity, antibodies directed against hydrophobic region of the prion protein have been shown to prevent prion accumulation in infected cells (White et al., 2003). The deletion of the 112-119 region of PrP^{c} results in resistance to infection *in vitro*, suggesting that this region is required for conversion (Norstrom and Mastrianni, 2005). Studies have shown that the hydrophobic region of PrP^{c} interacts with PrP^{sc} or the 106-126 peptide resulting in a loss of function of PrP^{c} (Brown, 2000b).

Substitution of the hydrophobic residues in the 106-126 peptide results in a reduction in toxicity (Jobling et al., 1999), suggesting that the hydrophobic region has a role in toxicity. A study using neuronal cells treated with recombinant prion protein have also shown that the hydrophobic region is required for prion toxicity (Simoneau et al., 2007). However removal of Δ112-119 does not prevent aggregation of the prion protein in yeast (Norstrom and Mastrianni, 2005). The effect of the removal of Δ112-119 on aggregation in mammalian cells is unclear and need to be investigated further. However *in vivo* the deletion of the 105-125 region results in neonatal lethality in mice (Li et al., 2007b) suggesting that the removal of the hydrophobic region can also be toxic to cells. Neurodegeneration is also observed when a larger fragment is deleted (94-134) (Baumann et al., 2007). Our results demonstrated that PrP missing amino acids Δ112-119 was toxic. As deletion of this region results in neonatal lethality in mice this it is perhaps unsurprising that PrP Δ112-119 is toxic to cells even in the absence of manganese. We went on to test the toxicity of the mutant compared to WT MnPrP as there was a slight increase in toxicity when manganese is bound to the protein and the region has been shown to be important in

metal metabolism, being required for PrP^c internalisation in response to copper (Haigh et al., 2005). We did not observe any significant change in toxicity compared to WT MnPrP.

It may be interesting to investigate the removal of Δ106-126, this would allow us to investigate whether the region often used as an analogue of PrP^{sc} has any effect on the toxicity of MnPrP.

4.3.5 N-terminus

The N-terminal region is an unstructured flexible region of PrP. We have investigated two mutants that cover the N-terminal region spanning amino acids 23-112 and amino acids 23-171. As well as the previously discussed inherited mutations in the octarepeat region, the N-terminal region of the prion protein contains several other amino acid mutations associated with inherited prion disease (Kitamoto and Tateishi, 1994), suggesting the region has a role in the conversion of the prion protein. However the reason for the lethality of these mutations remains unclear (Jones et al., 2006).

Our studies showed that the N-terminal region of PrP was highly toxic once bound to manganese and was significantly more toxic than apoPrP (23-112) suggesting the toxicity was due to metal binding. The N-terminus of PrP (23-112) contains all the metal binding regions previously discussed, but interestingly was more toxic than wild type MnPrP suggesting that manganese binding has a significant effect on the N-terminal mutant. As yet there have been there have been no studies investigating the structure of this fragment once bound to manganese. Studies investigating the structure of the native N-terminus have demonstrated that it is structured at higher pH (Zahn, 2003) and flexible at low pH (Donne et al., 1997; O'Sullivan et al., 2007). Low pH conditions been shown to cause the protein to adopt a higher β-sheet content.

Studies of the metal binding to the N-terminal region have revealed metal binding confers structure on the N-terminus (Jones et al., 2004). However in order to fully understand the effect of metal binding on the N-terminal fragment further work

would need to be done. A fuller understanding of the structure the N-terminal fragment, once bound to manganese, may lead to a better understanding of the reasons for the toxicity of this protein.

The N-terminus is thought to be important for the formation of higher order aggregates such as oligomers and fibrils. Work using N-terminally truncated recombinant protein (amino acids 90-231) revealed it formed smaller aggregates when compared to the wild type protein (Frankenfield et al., 2005). Work using recombinant prion protein missing amino acids 34-133 also showed a significant reduction in aggregation, which was associated with a decline in conversion efficiency in a cell free assay, although this was dependent upon glycosolation (Lawson et al., 2001). More recent work assessing the effect of pathogenic mutations on the prion protein structure has found that several mutations expose the N-terminus (Yin et al., 2007). The N-terminus has been shown to bind glycosaminoglycans (GAGs) (Pan et al., 2002), which have been shown to be required for cellular PrP^{sc} incorporation (Shaked et al., 2001) and are able to reconstitute the infectivity of prion rods (Hijazi et al., 2005). The mutations in the N-terminus result in the exposure of GAG binding sites and the resultant GAG binding leads to increased aggregation of the prion protein (Yin et al., 2007). It may be that as the C-terminus is not present in our N-terminal prion protein the regions involved in GAG binding are exposed in the manganese bound N-terminus allowing GAG binding and increased aggregation. Aggregation of the prion protein may lead to an increase in the toxicity of the protein. However further work is required to assess the aggregation of the N-terminal prion protein when bound to manganese and whether the regions involved in binding GAGs are exposed. It may be worth investigating if inhibiting GAG binding results in a reduction in toxicity.

We also investigated a longer region of the N-terminus of PrP spanning residues 23-171, in contrast to 23-112 we found that this region of the protein was toxic to cells in the absence of manganese, suggesting that toxicity observed once manganese was bound was not due to manganese alone but some other mechanism. It has been shown that an N-terminal fragment of human PrP spanning amino acids 23-144 spontaneously converts from a monomeric unordered state to a fibrillar form. This requires the presence of residues 130-148 (Kundu et al., 2003), so offers no

explanation for the toxicity of the N-terminal mutant 23-112, however it may offer an explanation for the intrinsic toxicity of the fragment 23-171, which contains residues 130-148. Further work has suggested that the addition of helix 1 (amino acids 144-156) to the N-terminal fragment causes aggregation to occur much more rapidly suggesting that this helix is involved in the conversion of the protein (Kundu et al., 2003). As the fragment 23-159 is readily able to aggregate (Watzlawik et al., 2006) it may also be that our slightly longer fragment also retains these properties, however further work would need to investigate the aggregation of 23-171.

4.3.6 C-terminus

The C-terminus region has been shown to contain the only known histidine mutation involved in inherited forms of the disease, at amino acid 187 (Cervenakova et al., 1999) in addition to several other mutations associated with in GSS. Cleaved fragments of the C-Terminus have also been found in the brains of people suffering with CJD, and these fragments have been shown to be PK resistant (Zou et al., 2003). The C-Terminus consists of 3 α- helices, one of which was recently been shown to be toxic to cells in the presence of copper, although the reason for copper requirement was not clear (Thompson et al., 2000).

Mice expressing a C-terminal fragment of the prion protein (amino acids 121-231) instead of wild-type prion protein die from massive neuronal degeneration within weeks of birth (Shmerling et al., 1998). This region of the protein has been shown to form soluble oligomers and amyloid fibrils under denaturing conditions despite the absence of the amyloidogenic core of PrP (Martins et al., 2006). Experiments using the N-terminally truncated recombinant prion protein have revealed that 121-231 is toxic to neuronal cells and that this toxicity is enhanced in PrP null cells. This region of the prion protein has been found contain several disease related mutations and these mutations also enhance the toxicity of 121-231 to neuronal cells (Daniels et al., 2001). However the toxicity of the fragment is lost when amino acids 113-120 are added to the fragment suggesting this region of the protein has a role in mediating the toxicity of the C-terminus (Daniels et al., 2001). Our results also confirmed that the 113-231 fragment of PrP was not toxic to neuronal cells when not bound to

manganese, and appeared to be one of the least toxic apoPrP fragments we analysed, as it caused almost no cell death even at the highest doses.

The C-terminus is a highly structured part of the prion protein, and is not generally thought to be involved in metal binding. Our study has shown that this protein is significantly less toxic than the WT prion. The decrease in toxicity is expected, as there are no metal binding sites known to bind manganese in the construct, however the difference in toxicity is not as great as the difference between the null construct and the WT protein. The reason for this is not clear it may be that the removal of the N-terminus changes the structure of the protein which may allow limited manganese binding, which would increase the toxicity of the protein. There is evidence for at least one functional binding motif for metals in the C-terminus. It has been shown that copper can bind to a histidine residue at 187 at pHs approaching neutrality (Brown et al., 2004), this binding site is also be able to bind manganese. As this region is highly structured it is possible that metal binding in this region is important in structure. This histidine certainly has an important role *in vivo*, as it is the only histidine known to be associated with the inheritable prion disease GSS (Cervenakova et al., 1999).

The study of regions of the prion protein involved in toxicity has revealed a complex process, and requires further work in order to elucidate the mechanisms that are involved, giving a better understanding of the why changes in toxicity occur in the mutants. Investigating changes to the mutants once bound to manganese such as structural and aggregatory changes and in may be the first step in a fuller understanding of the mechanisms underlying the changes in toxicity we observed. Whilst many groups use fragments of PrP to research prion toxicity it is often unclear whether removal of large parts of the protein may change its structure, stability and its ability to bind metals, this means work using fragments does not accurately reflect an *in vivo* situation. However work undertaken with the mutants where residues were simply replaced, such as the null mutant, maybe provide a model more relevant to the *in vivo* situation. Using these mutants we demonstrated the importance of the presence of both the fifth site and octarepeat as without the metal binding sites in the octarepeat region the protein became more toxic to the cells, possibly due to destabilisation of the protein.

It is clear from our model that manganese binding plays a role in modulating the toxicity of the prion protein. As there is some evidence the octarepeat region is not involved in modulating prion toxicity (Bonetto et al., 2002), it is not surprising this region alone with no fifth site did not increase the toxicity of the protein. However the fifth site alone, without the protective effect of the octarepeat region was highly toxic, suggesting that whilst the octarepeat alone does not increase toxicity it is required to protect the protein. Metal binding domains are unlikely to be the only regions involved in prion protein conversion, as regions such as the hydrophobic region have been shown to be linked to the conversion and toxicity of the protein (Jobling et al., 1999; Norstrom and Mastrianni, 2005).

It is unclear why metal binding changes the toxicity of the protein although the presence of metal ions has been shown to result in prion aggregation suggesting aggregation may play a role (Giese et al., 2004; Kim et al., 2005; Levin et al., 2005). In the absence of the octarepeat region metals have been shown to destabilise the protein and it may be that manganese destabilises the protein, especially when mutations are present in the protein or the protein has been cleaved. Studies have shown that manganese binding alters the structure of full-length PrP increasing the amount of β-sheet (Brown et al., 2000), suggesting that manganese alters the structure, but does not necessarily destabilise the protein, when no mutations are present. The increased level of β- sheet may result in aggregation which is linked to toxicity (Novitskaya et al., 2006). The change in structure may also result in regions of the protein being revealed such as GAG binding sites, which are required for conversion. Structural change may also result in changes to the prion protein's interactions with other proteins, several studies have investigated whether PrP^{c} binds to s receptor and PrP^{c} has been shown to bind the laminin receptor resulting in PrP^{c} internalisation (Saborio et al., 2001). In infected cells reduction of laminin receptor expression using siRNA has been shown to clear infection (Leucht et al., 2003) suggesting that PrP^{sc} is still able to bind the receptor despite the structural changes, however the downstream effects of the binding are likely to be very different.

In order to take this work forward metal binding, structural and aggregation studies should be carried out on the protein. This would allow us to tie together changes in toxicity with alterations in the metal binding, structure and aggregation, both known

factors in the toxicity of the prion protein. This would give us a more complete picture of the effect of manganese on the prion protein and allow us to better understand the role of aberrant metal binding in prion toxicity.

5. Cell Signalling in Prion Disease

Prion diseases cause massive neuronal cell death in the latter stages of disease leading to the brains classical spongiform appearance. This cell death is widely agreed to be caused by apoptosis. However, the cell signalling pathway leading to apoptosis remains unclear.

Several studies have identified neuronal apoptosis both *in vitro* (Forloni et al., 1993; Thellung et al., 2002) and *in vivo* (Fairbairn et al., 1994; Giese et al., 1995; Gray et al., 1999) using a variety of markers including caspase 3 activation and DNA fragmentation. Several studies have suggested that caspase 3 activation may be due to p38 activation and inhibition of p38 activation has been shown to prevent apoptosis *in vitro* (Thellung et al., 2002). However, other studies have shown that caspase 3 activation is p53 mediated, although inhibition of p53 activation is not sufficient to prevent disease progression (Engelstein et al., 2005).

Endoplasmic reticulum stress has also been suggested to play a role in neuronal cell death in prion disease. Some studies have shown caspase 12, an ER resident caspase, activated by ER stress, is activated in response to PrP *in vitro* (Hetz et al., 2003b). However, more recent studies have suggested that ER stress does not play a crucial role in cell death in prion diseases (Unterberger et al., 2006). Other studies have suggested that calcium release from the ER activates the mitochondrial apoptotic pathway (Ferreiro et al., 2008a; Ferreiro et al., 2008b).

Many studies have used the 106-126 peptide to investigate cell death in prion disease (Corsaro et al., 2003; Ferreiro et al., 2008a; Ferreiro et al., 2008b; Forloni et al., 1996; Saez-Valero et al., 2000; Thellung et al., 2002). In this study we intend to use the MnPrP model to investigate cell signalling caused by PrP, as whilst we have demonstrated that MnPrP is toxic to neuronal cells, the signalling mechanisms that result in cell death have not been investigated.

This study aims to further characterise the model of toxicity established in chapter 1 by investigating the mechanism of cell death induced by MnPrP. We have used a

variety of techniques to confirm that cell death is due to apoptosis and that this may be mediated by p38 phosphorylation and caspase 3 cleavage.

We have investigated mechanisms by which SMB cells are able to survive chronic infection. We have confirmed that SMB cells are not undergoing apoptotic cell death whilst in culture. There is also no evidence of caspase 3 or p38 up-regulation in SMB cells. However we have found evidence of high levels of caspase 12 expression suggesting there is ER stress in SMB cells. We investigated signalling molecules important in neuronal survival in SMB cells and found ERK phosphorylation was increased in SMB cells and that this increase was involved in cell survival. An increase in ERK phosphorylation was observed in F21 cells treated with MnPrP suggesting that ERK may be important in protecting cells from prion toxicity

5.1 F21 signalling in the presence of MnPrP

5.1.1 Cell death signalling

DNA laddering was used to establish whether the toxicity we had observed was due to apoptosis. DNA laddering occurs as the DNA is broken down, during apoptosis, in the cell in a controlled manner, leading to DNA fragments increasing in size by 200bp. F21 cells were treated for 48h with increasing concentrations of MnPrP. The cellular DNA was extracted and run on a 1.5% agarose gel. DNA laddering could clearly be seen in lanes treated with 40 µg/ml and 80 µg/ml MnPrP. This DNA laddering mimicked the DNA laddering observed in F21 cells treated for 24h with stuarosporine, which is known to induce apoptosis. However, in F21 cells treated with MnPrP for 24h DNA laddering was not easily visible (figure 5.1).

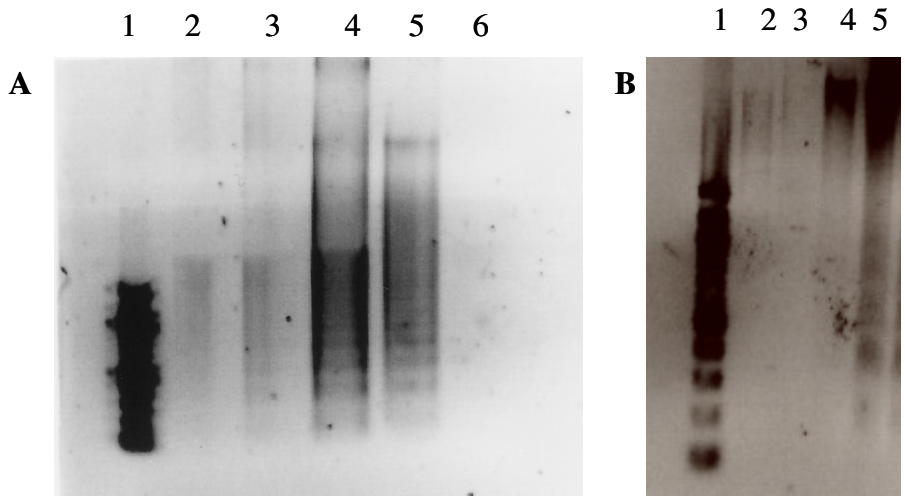


Figure 5.1: DNA laddering in F21 cells treated with MnPrP. A: F21 cells were treated for 48h with increasing concentrations of MnPrP, cellular DNA was then extracted and separated on a 1.5% agarose gel. Lane 1: 100bp ladder, lane 2: untreated control, lane 3: 20µg/ml MnPrP, lane 4: 40µg/ml MnPrP, lane 5: 80µg/ml MnPrP, lane 6: Manganese control. B: F21 cells were treated for 24h with increasing concentrations of MnPrP and a stuarosporine control. Cellular DNA was then extracted and separated on a 1.5% agarose gel. Lane 1: 100bp ladder, lane 2: control, lane 3: 80µg/ml MnPrP, lane 4: 160µg/ml MnPrP, lane 5: 200µM staurosporine.

To further confirm that MnPrP causes apoptosis, we investigated whether MnPrP causes caspase 3 cleavage. Several previous reports have shown caspase 3 activation both in cells treated with the 106-126 peptide and in the brains of mice infected with scrapie. Caspase 3 activation is one of the final steps in the induction of apoptosis. Cells were treated for 4, 8, 16 and 24h with 80 µg/ml MnPrP and caspase 3 cleavage was assessed using Western blotting. Caspase 3 cleavage was seen after 16h of treatment with MnPrP and was still visible after 24h (figure 5.2).

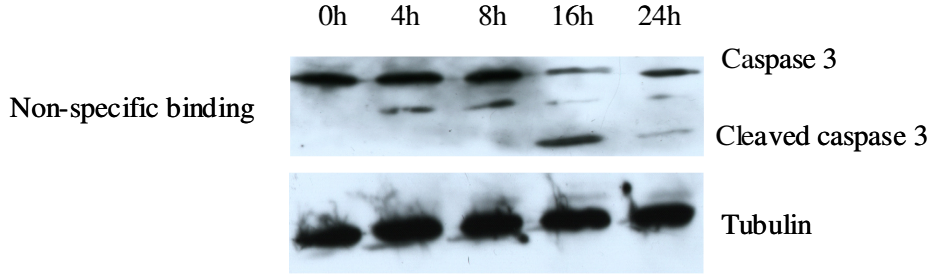


Figure 5.2: Caspase 3 cleavage in F21 cells treated with MnPrP. F21 cells were treated with 80µg/ml MnPrP for varying lengths of time. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. Caspase 3 expression and cleavage was assessed using western blotting. Lane 1: 0h, Lane 2: 4h, Lane 3: 8h, Lane 5: 16h, Lane 6: 24h.

As both p53 and p38 have been implicated in apoptosis in prion disease, we investigated the expression of p53 and p38 phosphorylation in F21 cells treated for 4, 8, 16, and 24h with 80 µg/ml MnPrP. There was a significant decrease in p53 expression (figure 5.3).

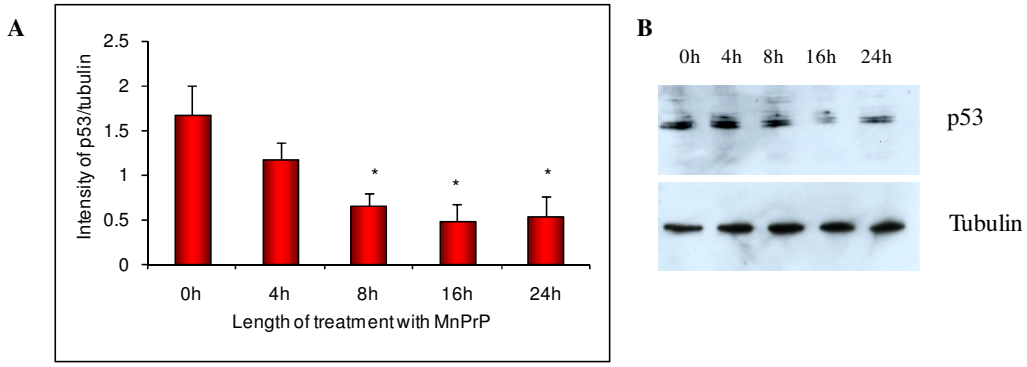


Figure 5.3: Expression of p53 by F21 cells treated with MnPrP. F21 cells were treated with 80µg/ml MnPrP for varying lengths of time. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. p53 expression was assessed by western blotting. A: Intensity was quantified using Image J and normalised using tubulin intensity N=4 error bars are SEM *p>0.05 as tested by students T-test. B: Lane 1: 100bp ladder Lane 2: 0h, Lane 3: 4h, Lane 4: 8h, Lane 5: 16h, Lane 6: 24h.

There was evidence of p38 phosphorylation in cells treated with MnPrP for 16h (figure 5.4) this is in line with toxicity studies in previous reports that have shown that p38 activation is required for the activation of caspase 3 in the presence of PrP peptides.

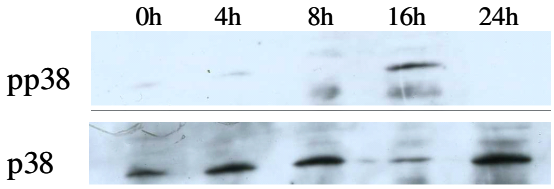


Figure 5.4: Expression of pp38 by F21 cells treated with MnPrP. F21 cells were treated with 80µg/ml MnPrP for varying lengths of time. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. pp38 and p38 expression and activation was assessed using western blotting. Lane 1: 0h, Lane 2: 4h, Lane 3: 8h, Lane 5: 16h, Lane 6: 24h.

We also investigated other pathways that could be up-regulated in cells treated with MnPrP. In order to assess the role that the ER might play in MnPrP toxicity we investigated BiP. BiP has previously been shown to bind abnormal forms of PrP (Jin et al., 2000) and is up-regulated in response to ER stress, providing a protective

effect (Rao et al., 2002). BiP has also been shown to be up-regulated in response to PrP 106-126 (Ferreiro et al., 2008a). We found that treatment for 24h with MnPrP did not lead to an increase in BiP expression in F21 cells (figure 5.5).

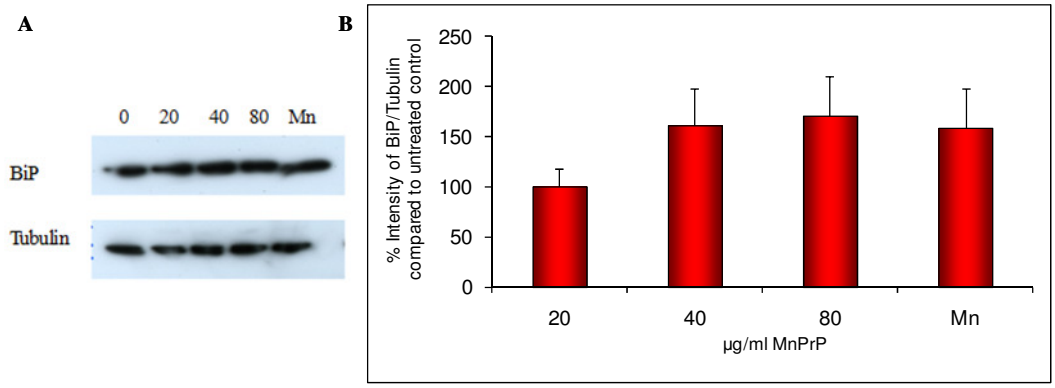


Figure 5.5: Expression of BiP by F21 cells treated with MnPrP. F21 cells were treated with varying concentrations of MnPrP for 24h. Cellular protein was then extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. BiP expression was assessed by Western blotting and normalised to tubulin. Intensity was assessed using Image J. n=5 no points are significant as tested by students T-Test.

5.1.2 Cell Survival Signalling.

We also investigated several signalling molecules thought to be important in cell survival signalling. We investigated AKT and ERK both thought to be involved in cell survival signalling in response to neuronal growth factors. AKT has been shown to promote neuronal survival via PI3K. PI3K is activated in response neuronal growth factors and this signalling leads to a wide variety of responses including inhibition of pro-apoptotic molecules such as p53 (Yamaguchi et al., 2001) and activation of transcription factors involved in cell survival such as NF-κB. ERK is a member of the MAPK group of signalling molecules, and was first found to be important in neuronal survival in response to neurotrophins (Xia et al., 1995). It has also been shown to be important in cell survival in response to injury in certain circumstances. More recent evidence has shown that ERK can also be involved in regulation of apoptosis (Murray et al., 1998).

We found no change in the levels of AKT activation in F21 cells treated for 24h with MnPrP (figure 5.6). We also found no change in the activation of ERK after 24h treatment with MnPrP (figure 5.7). However after 48h we found a significant

increase in activation (figure 5.7). This may be due to the small surviving population being protected by ERK up-regulation.

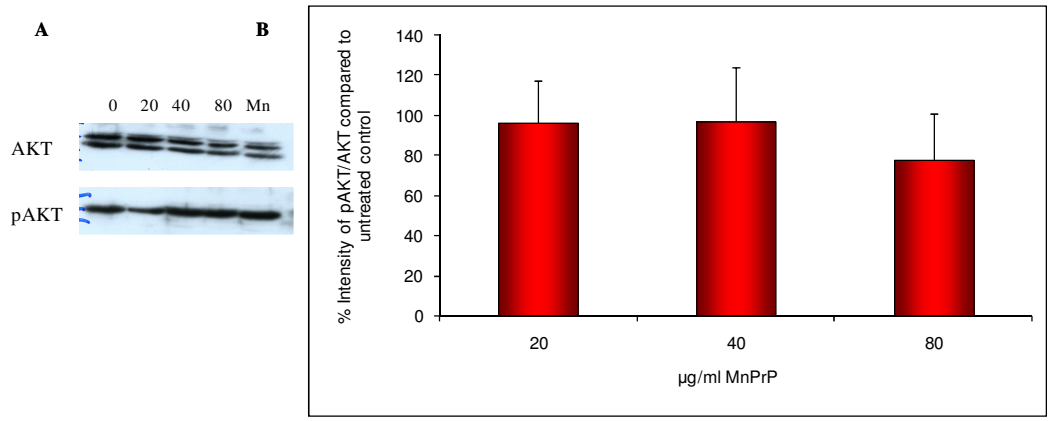


Figure 5.6: Expression of pAKT by F21 cells treated with MnPrP. A: F21 cells were treated with varying concentrations of MnPrP for 24h. Cellular protein was then extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. AKT phosphorylation was assessed by Western blotting and normalised to AKT. B: Intensity was assessed using Image J. n=5

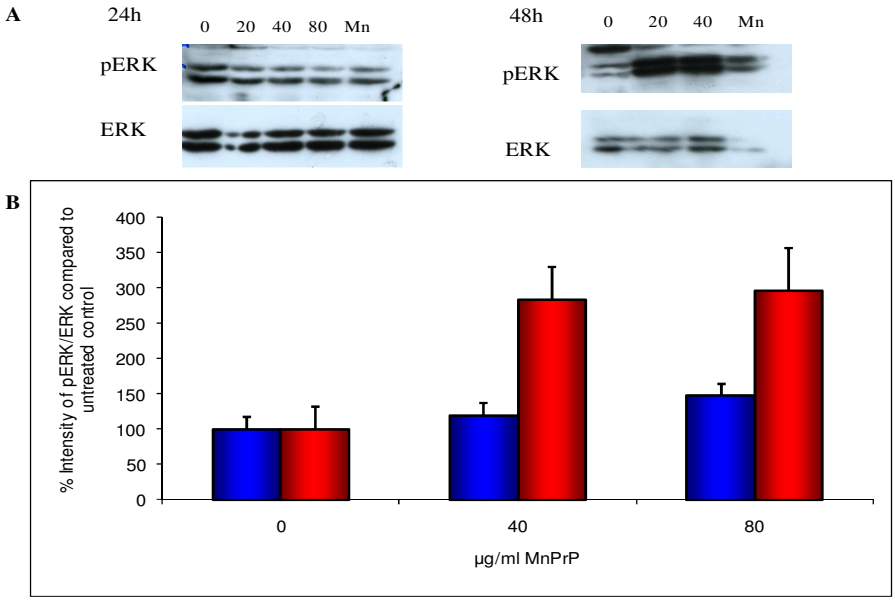


Figure 5.7: Expression of pERK by F21 cells treated with MnPrP. A: F21 cells were treated with varying concentrations of MnPrP for 24h or 48h . Cellular protein was then extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. ERK phosphorylation was assessed by Western blotting and normalised to ERK. B: Intensity was assessed using Image J. Blue bars: cells were treated for 24h with MnPrP n=7 no points are significant students T-Test. Red bars: cells were treated for 48h with MnPrP n=5 both 40 and 80µg/ml are significant $p<0.05$ compared to control.

As ERK was activated in response to MnPrP we investigated the expression of 14-3-3 a possible upstream activator of ERK that is found in high concentrations in the

cerebrospinal fluid (CSF) of patients suffering from prion disease (Hsich et al., 1996). There was no increase in 14-3-3 expression at 24h (figure 5.8) however further investigations at a later time point maybe more useful in determining whether it regulates ERK in response to MnPrP.

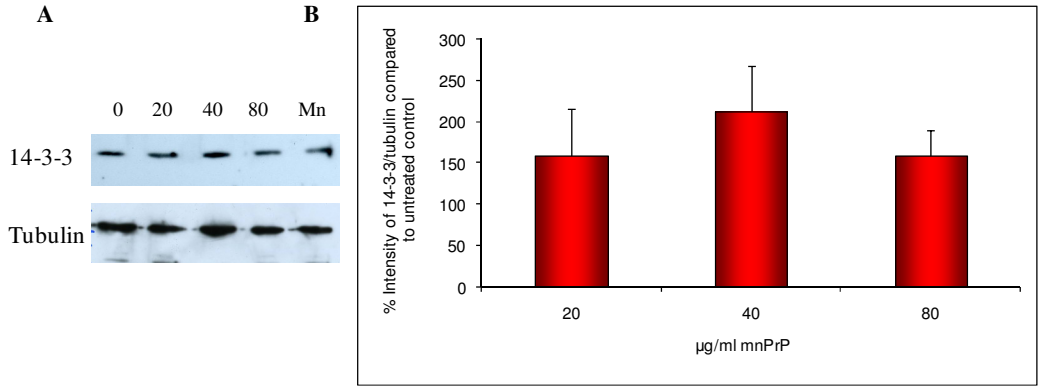


Figure 5.8: Expression of 14-3-3 by F21 cells treated with MnPrP. A: Expression of 14-3-3 in F21 cells treated with varying concentrations of MnPrP for 24h. Cellular protein was extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. 14-3-3 expression was assessed by Western blotting and normalised to tubulin. B: Intensity was assessed using Image J n=8 no points are significant when compared to control as assessed by students T-Test.

5.2 SMB cells

5.2.1 Cell Death Signalling

Having earlier established that MnPrP induced apoptosis in SMB cells (chapter 3) we went on to investigate the effects of scrapie infection on SMB cells. Initially we investigated cell signalling molecules involved in cell death. In order to confirm that there was no evidence of low level apoptosis in the SMB cell line we used DNA laddering. We found no evidence of laddering in SMB cells (Figure 5.9b). We also investigated whether caspase 3 and 9 were activated in SMB and SMB-PS cells and found no evidence of activation in either cell line (figure 5.9a).

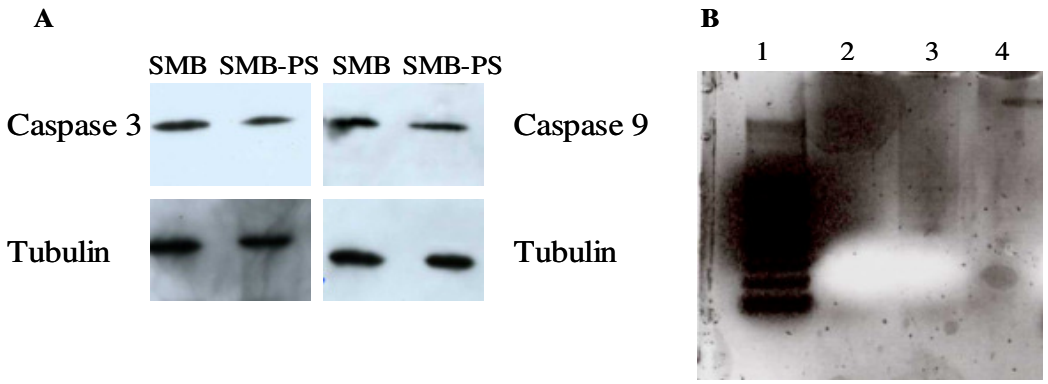


Figure 5.9: Caspase 3 and 9 cleavage and DNA laddering in SMB and SMB-PS cells. A: SMB and SMB-PS cells were plated and left to adhere overnight. Cellular protein was extracted using RIPA buffer and proteins separated on an SDS-PAGE gel. Caspase 3 and caspase 9 expression and activation were assessed using western blotting. Lane 1: SMB, Lane 2: SMB-PS. B: DNA laddering Lane 1: F21 untreated, lane 2: F21 80µg/ml MnPrP, lane 3: SMB cells.

We investigated the levels of caspase 3 and 9 expression in both SMB and SMB-PS cells there was no evidence of an increase in either caspase 3 or caspase 9 expression in SMB cells when compared to SMB-PS cells (Figure 5.10).

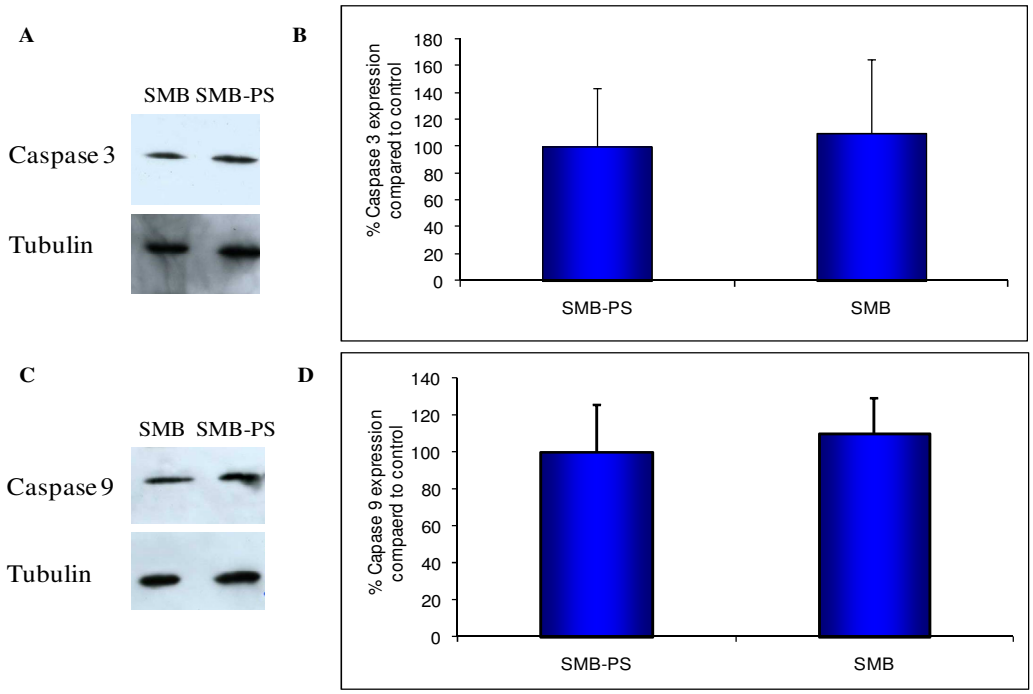


Figure 5.10: Caspase 3 and 9 expression in SMB and SMB-PS cells. A & C: SMB cells were plated out and left overnight to adhere. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. Caspase 3 (A) or caspase 9 (C) expression was assessed by western blotting. A: Lane 1: SMB, Lane 2: SMB-PS. B & D: Intensity was quantified using Image J and normalised using tubulin intensity n= at least 4 error bars are SEM *p>0.05 as tested by students T-test.

We also assessed other signalling molecules associated with apoptosis in prion disease including p38. p38 phosphorylation was investigated by western blotting and no difference was observed between SMB and SMB-PS cells (figure 5.11).

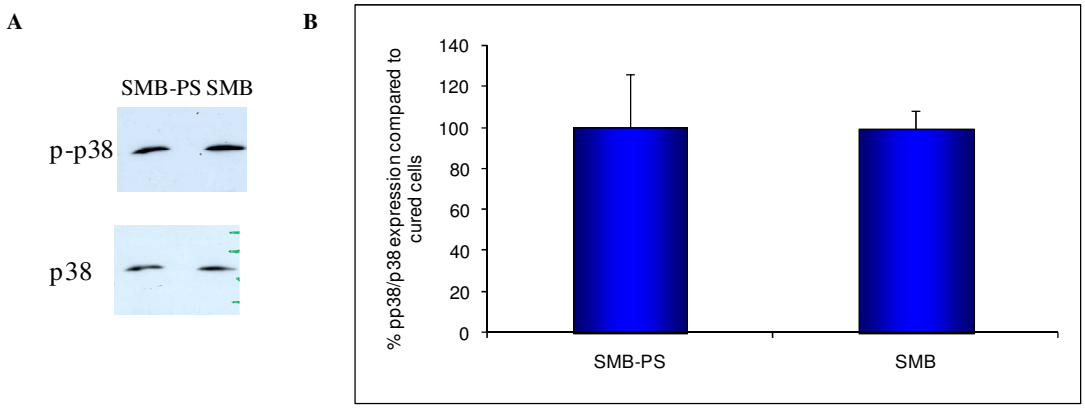


Figure 5.11: pp38 expression in SMB and SMB-PS cells. A: SMB cells were plated out and left overnight to adhere. Cellular protein was extracted using RIPA buffer and proteins were separated on a SDS-PAGE gel. pp38 and p38 expression was assessed using western blotting. A: Lane 1: SMB, Lane 2: SMB-PS. B: Intensity was quantified using Image J and normalised using p38 intensity N=6 error bars are SEM. p>0.05 as tested by students T-test.

5.2.2 Endoplasmic reticulum stress

As several reports have indicated that ER stress may have an important role in prion disease, we investigated the expression of caspase 12, an ER resident caspase. SMB cells had very high caspase 12 expression levels, whilst in cured cells caspase 12 expression was almost undetectable (figure 5.12).

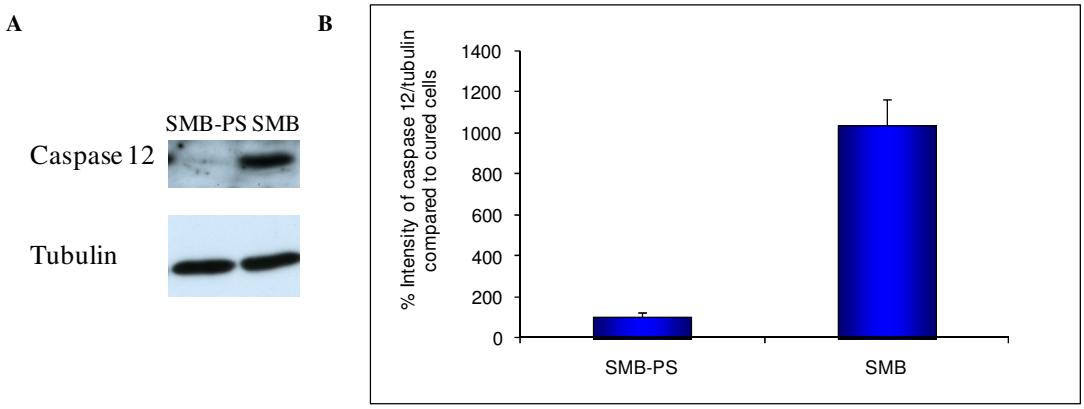


Figure 5.12: Caspase 12 expression in SMB and SMB-PS cells. A: SMB cells were plated out and left overnight to adhere. Cellular protein was extracted using RIPA buffer and proteins were separated on an SDS-PAGE gel. Caspase 12 and tubulin expression was assessed using western blotting (upper band is non-specific binding). A: Lane 1: SMB, Lane 2: SMB-PS. B: Intensity was quantified using Image J and normalised tubulin intensity N=9 error bars are SEM. $p<0.05$ as tested by students T-test.

We also investigated the expression of BiP. BiP is a chaperone that has been shown to bind to abnormal forms of PrP. Interestingly, there was no evidence of increase in expression of BiP in SMB cells (figure 5.13).

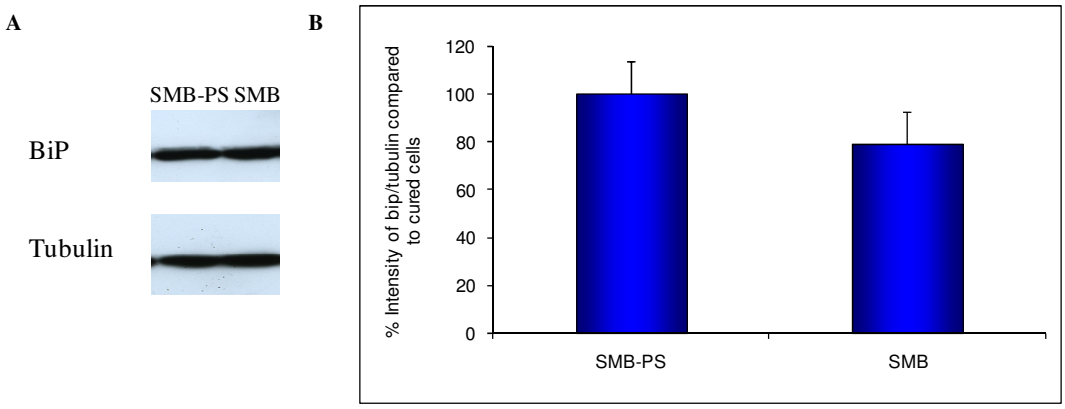


Figure 5.13: BiP expression in SMB and SMB-PS cells. A: SMB cells were plated out and left overnight to adhere. Cellular protein was extracted using RIPA buffer and proteins separated on a SDS-PAGE gel. BiP expression was assessed using western blotting. A: Lane 1: SMB, Lane 2: SMB-PS. B: Intensity was quantified using Image J and normalised to tubulin intensity N=11 error bars are SEM. $p>0.05$ as tested by students T-test.

5.2.3 Cell Survival Signalling

In order to identify signalling molecules that might enhance survival in SMB cells, we investigated molecules ERK and AKT that have a role in neuronal survival in the normal brain.

There was no difference in AKT phosphorylation between SMB and SMB-PS cells (figure 5.14 a, b), however ERK phosphorylation was significantly higher in SMB cells (figure 5.14 c, d) compared to SMB-PS cells.

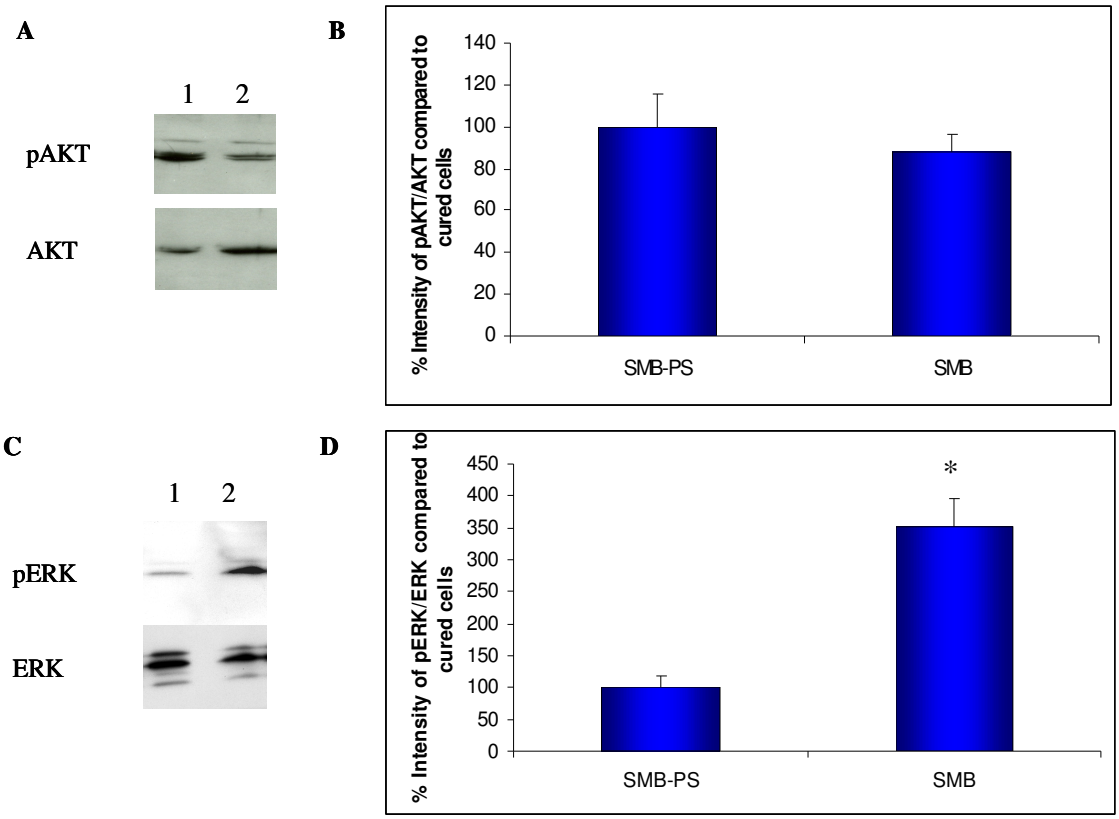


Figure 5.14: pERK and pAKT expression in SMB and SMB-PS cells. A & C: SMB cells were plated out and left overnight to adhere. Cellular protein was extracted using RIPA buffer and proteins were separated on a SDS-PAGE gel. pAKT (A) or pERK (C) expression was assessed using western blotting. A: Lane 1: SMB, Lane 2: SMB-PS. B: Intensity was quantified using Image J and normalised using either AKT (B) or ERK (D) intensity. n=3 error bars are SEM *p<0.05 as tested by students T-test.

To investigate whether this up-regulation was functional, ERK phosphorylation was blocked using a specific MEK inhibitor PD98059 (Figure 5.15a). MEK is the sole upstream activator of ERK. SMB cells treated with the inhibitor showed a significant loss in viability when compared to the control, this was not observed in SMB-PS cells, suggesting this up-regulation is functional (Figure 5.15b).

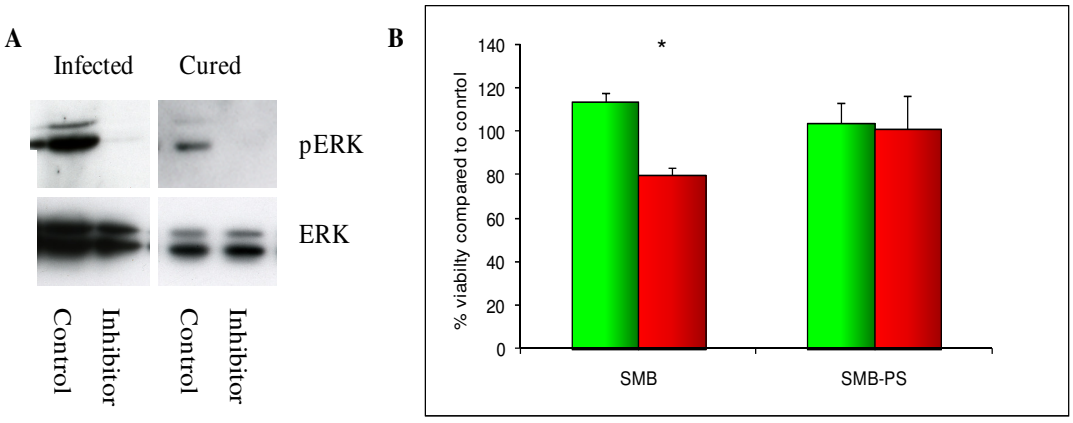


Figure 5.15: The effect the inhibition of ERK activation on SMB and SMB-PS survival. A: SMB and SMB-PS cells were treated for 1h with a specific MEK inhibitor PD98059 and the cellular protein was extracted using RIPA buffer. Western blotting was used to determine the expression of pERK and ERK. B: SMB and SMB-PS cells were plated in media 199 and left to adhere overnight. Cells were then treated with a specific MEK inhibitor PD98059 for 16h viability was assessed using an MTS assay. n=3. *p= 0.03 (Students T Test). Error bars SEM. (DMSO included as it was used to solubilise the inhibitor)

As unpublished work from our lab has shown that SMB cells have increased resistance to certain types of oxidative stress, we postulated that ERK may be important in protecting cells from oxidative stress caused by infection, however, treatment with PD980959 had no effect on basal levels of ROS in SMB or SMB-PS cells (figure 5.16).

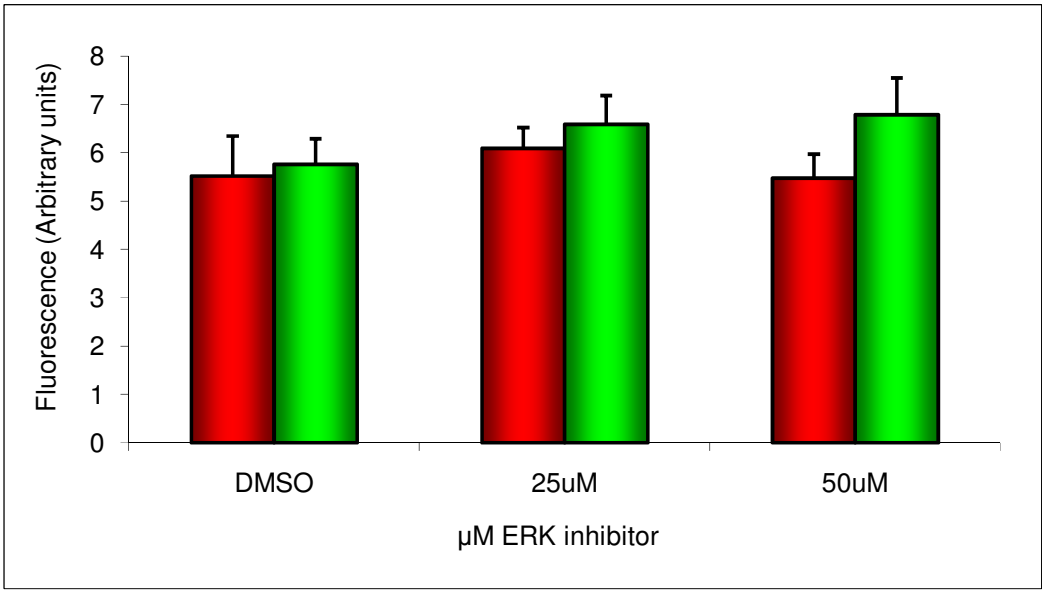


Figure 5.16: The effect the inhibition of ERK activation on SMB and SMB-PS cellular ROS expression. SMB (red) and SMB-PS (green) cells were plated in serum free media and left to

adhere overnight. Cells were then treated with a specific MEK inhibitor PD98059 and ROS were assessed using DCDFDA, a probe that is cleaved and creates a fluorescent signal in response to ROS. n=4. No points are significant (Students T Test). Error bars SEM. (DMSO included as it was used to solubilise the inhibitor)

In order to investigate the upstream signalling mechanisms that may lead to up-regulation of ERK, we decided to examine the expression of 14-3-3. 14-3-3 has been found in the CSF of patients with CJD and is thought to be early marker of disease (Hsich et al., 1996). It is also one of the potential activators of ERK. Previous research has shown inhibition of certain homolog's of 14-3-3 can lead to ERK suppression and an increase in activation of apoptotic pathways (Xing et al., 2000). Our work, using a pan 14-3-3 antibody has found that 14-3-3 is up-regulated in SMB cells, this up-regulation may play a role in ERK activation (figure 5.17).

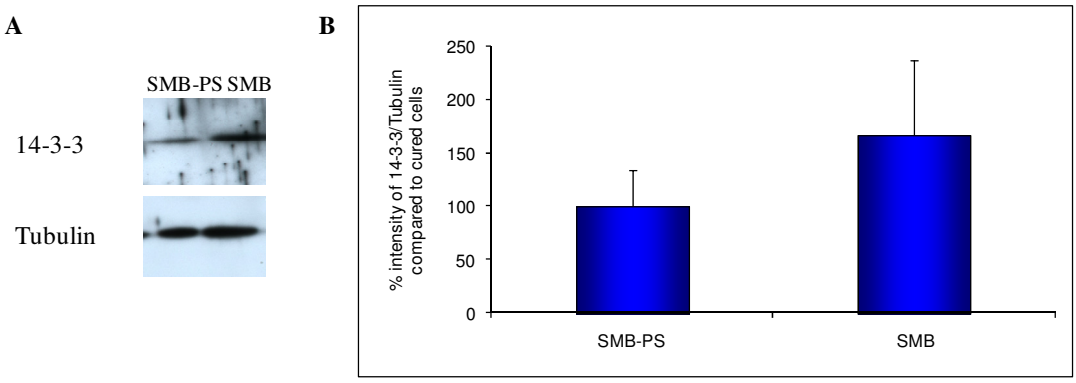


Figure 5.17: Expression of 14-3-3 in SMB cells or SMB-PS cells. A: Cellular protein was extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. 14-3-3 expression was assessed by Western blotting and normalised to tubulin. B: Intensity was assessed using Image J. n=4 $p<0.05$ as assessed by students T-Test.

As PKA is also a known activator of ERK, we also investigated its ability to regulate ERK. SMB cells were treated for 16h with a PKA inhibitor prior to viability analysis using a MTS assay. There was a slight but significant increase in viability in SMB cells treated with the PKA inhibitor for 24h (figure 5.18). However, there was no evidence of inhibition of ERK by western blotting, indicating that PKA is not involved in pERK activation but maybe involved in another pathway that is responsible for SMB survival (Figure 5.19).

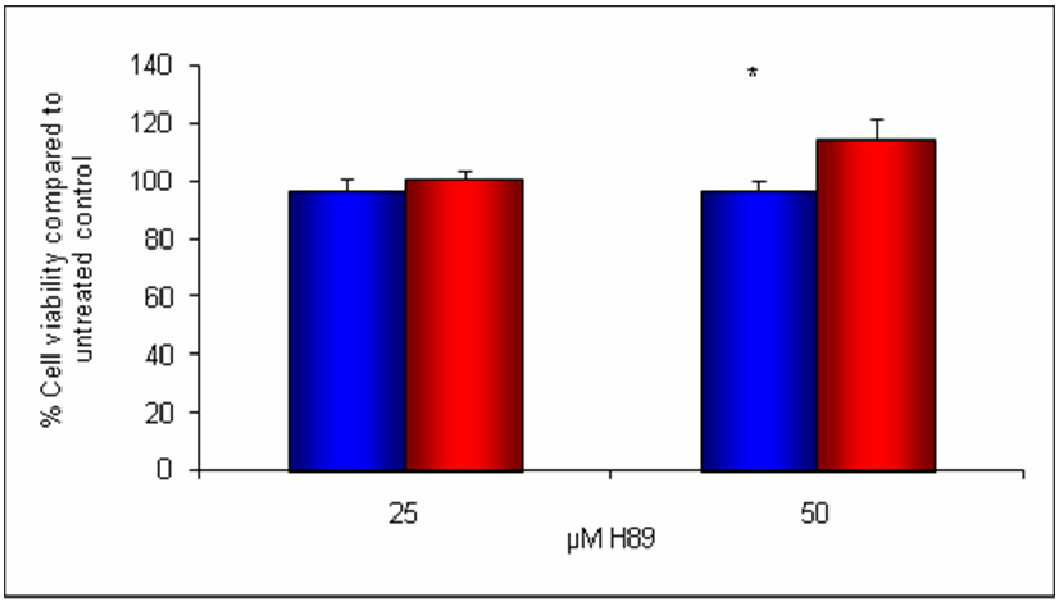


Figure 5.18: Effect of PKA inhibition on SMB and SMB-PS survival. SMB (Red) and SMB-PS (blue) cells were treated for 24h with varying concentrations of H89 a specific PKA inhibitor, cell viability was assessed using an MTS assay. n=4. There was a significant increase in cell viability in SMB cells treated with 50μM H89 compared to untreated control, as assessed by students T-test $p<0.05$.

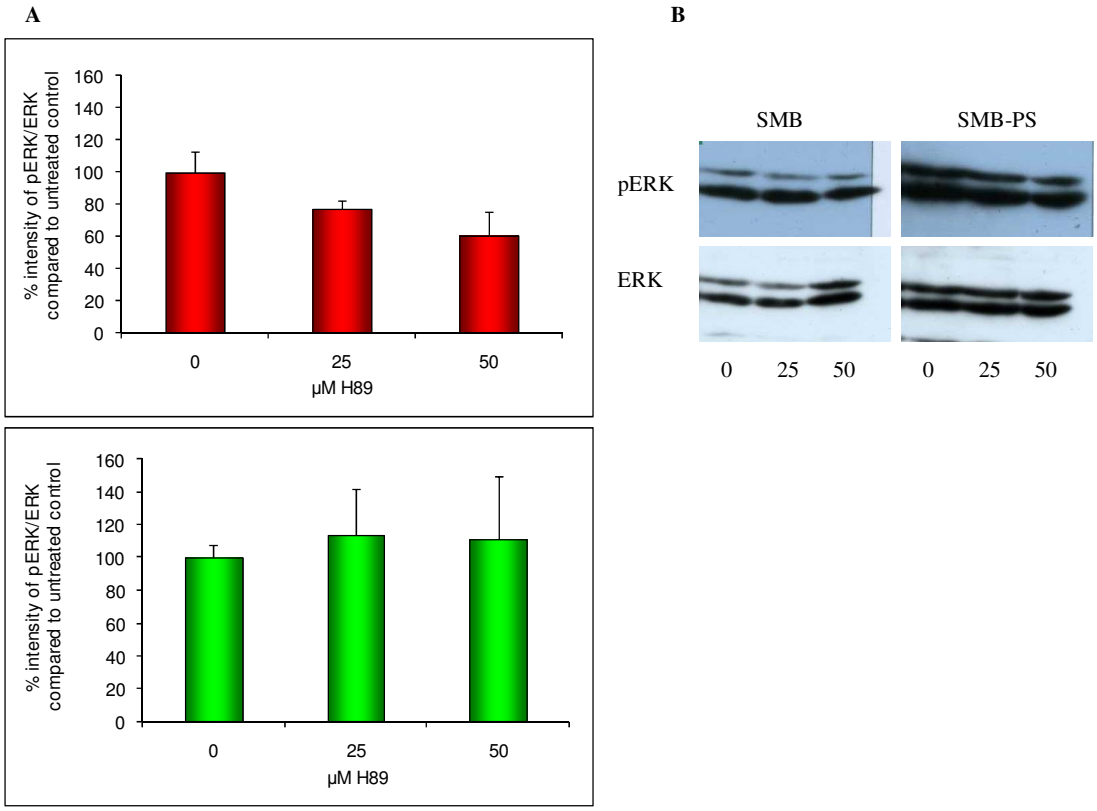


Figure 5.19: A: Expression of ERK in SMB and SMB-PS cells treated with a PKA inhibitor. Expression of pERK in either SMB cells (top) or SMB-PS cells (bottom) treated with H89 for 4h. Cellular protein was extracted using RIPA buffer and separated on a 12% SDS-PAGE gel. pERK expression was assessed by Western blotting and normalised to ERK. Intensity was

assessed using Image J. n=3. No points are significant when compared to control as assessed by students T-Test. B: Sample blots

5.3 Overview of Results

The table below provides an overview of the results, perhaps most striking of all the results is the up-regulation of ERK activation in both infected cells and surviving F21 cells. It is also clear that the cell death induced by MnPrP is apoptotic and is mediated by caspase 3 cleavage. There was no evidence of cell death in SMB cells suggesting that the infection carried by SMB cells is not enough to induce apoptosis. However earlier work had shown treatment with MnPrP was sufficient to induce apoptosis in similar manner to that observed in SMB cells.

	Signalling molecule	SMB Expression (compared to SMB-PS)	F21 Expression (Compared to untreated control)
Neuroprotective	ERK	↑	↑ (48h)
	AKT	↔	↔
	14-3-3	↑	↔
Endoplasmic Reticulum	BiP (GRP78)	↔	↔
	Caspase 12	↑	-
Cell Death	Caspase 3	↔	Cleaved (16h)
	Caspase 9	↔	-
	p-p38	↔	↑
	p53	-	↓

Table 4.1: A comparative table outlining the changes in expression in signalling molecules as assessed by western blotting. The SMB column shows the levels of expression of signalling molecules when compared to the cured cell line SMB-PS. The F21 column shows the expression of signalling molecules after treatment for 24h with MnPrP compared to control.

5.4 Discussion

5.4.1 Cell signalling and MnPrP

Previous studies have shown that prion diseases cause massive neuronal cell death (Fairbairn et al., 1994; Giese et al., 1995). *In vitro* results have demonstrated caspase 3 cleavage is at least partially responsible for cell death. Studies have shown that primary neuronal cells treated with PrP 106-126 underwent massive neuronal apoptosis characterised by DNA fragmentation and activation of caspase 3 (Carimalo et al., 2005), caspase 3 activation has also been observed in a neuronal cell line treated with PrP 106-126 (Corsaro et al., 2003).

Initial investigations have shown that MnPrP is toxic to several cell lines and to primary CGNs. In this chapter it was shown, using a variety of methods, that MnPrP causes apoptosis in both F21 and SMB cells. Initially DNA laddering was observed 48h after treatment with MnPrP. As caspase 3 has been shown to be required for DNA fragmentation (Janicke et al., 1998) we investigated whether caspase 3 was activated in response to MnPrP. We found that caspase 3 was activated at 16h after MnPrP treatment in F21 cells and was still visible at 24h. In SMB cells treated with MnPrP active caspase 3 was only seen at 16h. Further work in our laboratory has confirmed that caspase 3 is involved in cell death in our model as inhibition of caspase 3 using a specific caspase 3 inhibitor leads to an increase in cells viability in F21 cells treated with MnPrP (Uppington and Brown, 2008).

We further investigated possible upstream signalling pathways that may activate caspase 3. p53 is a signalling molecule involved in the regulation of cell cycle (Diller et al., 1990). p53 can activate caspase 3 in CGNs (Cregan et al., 1999). p53 has been shown to be involved in apoptosis in a variety of neurodegenerative diseases including Alzheimer's (Tamagno et al., 2003a) and Parkinson's (Martin et al., 2006). p53 has been implicated in apoptosis in prion disease (Engelstein et al., 2005). However inhibition of the p53 in hamsters infected with prion disease did not reduce incubation time, despite reducing caspase 3 expression (Engelstein et al., 2005). This implies that p53 mediated caspase 3 is not the sole mechanism of cell death in prion disease. PrP^c may have a role in p53 regulation, p53 studies indicate that p53-

dependent caspase 3 activation triggered is by the C1 fragment of PrP^c (Paitel et al., 2004; Sunyach et al., 2007) and this is dependent on PrP^c endocytosis (Sunyach and Checler, 2005).

In response to cellular stress p53 is up-regulated and its stability is increased. However in our model p53 expression significantly decreased suggesting that it is not involved in the activation of caspase 3 in our model. As there is evidence that p53-dependant caspase 3 activation can be triggered by PrP^c upon its endocytosis (Sunyach and Checler, 2005). We postulate that in our model PrP^c endocytosis is inhibited by MnPrP and therefore caspase 3 cannot be activated, via this pathway.

We investigated p38 activation as another possible mechanism for caspase 3 activation. Previous research using the 106-126 peptide had implicated p38 in caspase 3 activation (Thellung et al., 2002). Further work has shown that p38 is involved in caspase 3 activation in cells treated with PrP 90-231 (Corsaro et al., 2006; Villa et al., 2006). *In vivo* work has shown p38 is activated in the brains of hamsters infected with scrapie (Lee et al., 2005). We demonstrated that in our model p38 phosphorylation was visible 16h after treatment with MnPrP. As several previous studies have shown that PrP peptides mediate p38 phosphorylation leading to caspase 3 activation (Corsaro et al., 2006; Corsaro et al., 2003; Thellung et al., 2002; Villa et al., 2006), we believe that this pathway is at least one of the pathways responsible for cell death in our models. In order confirm its role in caspase 3 activation in our system, further work should be to inhibit p38 and investigate whether this reduced caspase 3 activation. Work using a fibrillar fragment of PrP, 90-231, has shown that inhibition of p38 activation leads to a reduction in apoptosis in cells treated with the fragment (Villa et al., 2006), further suggesting that the p38 pathway may be important in cell death in prion disease.

Studies of the therapeutic use of p38 inhibitors have shown they can reduce behavioural deficits in a mouse model of Alzheimer's disease (Munoz et al., 2007). As inhibition of the cell signalling mechanisms leading to cell death could be of use therapeutically, the upstream mechanism leading to p38 activation should be investigated to find other possible targets for therapeutic inhibition. As there is evidence that prion disease causes an increase in markers of oxidative stress in the

brain, it could be hypothesised that p38 is activated in response to the increase in ROS. Previous work has shown that treatment of neurones with PrP 106-126 leads to an increase in ROS and activation of p38 (Pietri et al., 2006). This activation of p38 is most likely triggered by NO production as this has been demonstrated to trigger activation of the p38 pathway in neurones (Moriya et al., 2000). This results in the translocation of Bax from the mitochondria leading to apoptosis (Ghatan et al., 2000). p38 can also be activated by hydrogen peroxide, studies using Aβ have shown that Aβ treatment leads to an increase in hydrogen peroxide production and activation of p38 (Tamagno et al., 2003b). Alpha synuclein and toxic forms of the prion protein have been shown to generate hydrogen peroxide *in vitro* (Tabner et al., 2005) suggesting that hydrogen peroxide may play a role in cell death in a variety of neurodegenerative diseases.

5.4.2 Cell signalling and infection.

Initial studies led us to believe that SMB cells carry a low level of prion disease, as the lysate from SMB cells is not toxic to either SMB cells or to F21 cells. However, we were interested in any modification in signalling that may have allowed cells to survive even a low level infection for such a long period of time, as previous research has shown changes in apoptotic signalling in cells treated with sub-lethal levels of 106-126 (White et al., 2001). Initially we wanted to confirm that there was no low level apoptosis in SMB cells. No DNA laddering in was observed in SMB cells, we also looked for caspase activation in both SMB and SMB-PS cells and found no evidence of activation in either cell line. Finally, as we have shown p38 activation in F21 cells treated with MnPrP, we investigated p38 activation in SMB and SMB-PS cells and found no evidence of activation or up-regulation in either cell line.

As SMB cells maybe under stress due to the infection they carry, we investigated the expression levels of several un-cleaved caspases in SMB and SMB-PS cells. We found no evidence of an increase in expression of caspase 3 and 9. However there was a large increase in caspase 12 expression in SMB cells, when compared to SMB-PS cells in which caspase 12 was almost undetectable. Interestingly, caspase 12 is an ER resident caspase, and as SMB cells have a large amount of misfolded

protein in the ER it may be an increase in caspase 12 expression in response to this accumulation. This is most likely a stress response and not protective to the cells. Further work would need to be undertaken to investigate whether the caspase 12 present in SMB cells is cleaved and if so, what downstream effects, occur including whether cleavage of caspase 12 leads to apoptosis. We also investigated whether BiP was up-regulated. BiP is an ER chaperone that has been shown to bind to misfolded PrP and mediate its degradation (Jin et al., 2000). However, no evidence of up-regulation could be found in SMB cells. Previous work on ER stress in prion disease has been contradictory, as some studies have shown active caspase 12 in N2A cells treated with PrP^{sc} (Hetz et al., 2003b), whilst, *in vivo* studies have shown that ER stress has a limited role in prion disease (Unterberger et al., 2006). More recent work has investigated the possibility that the ER releases calcium in response to PrP leading to mitochondrial mediated cell death (Ferreiro et al., 2008b), as scrapie infected cells appear to be under ER stress calcium release should be investigated in these cells.

In order to better understand survival pathways that may be activated in chronically infected cells we investigated ERK and AKT, two signalling molecules that have been shown to play an important role in neuronal survival. Both molecules respond to neurotrophins, which normally leads to survival. AKT signals via PI3K. ERK is a member of the MAPK family, and its sole upstream activator is MEK. Microarray studies have identified PrP^{c} as having a significant relationship with AKT, and MAPK signalling pathways (Satoh et al., 2008). There is evidence that AKT activation can be modulated by PrP^{c}, PrP knockout mice have reduced AKT activation and enhanced caspase-3 activation in response to cerebral ischemia (Weise et al., 2006). ERK activation is up-regulated in brains of hamsters infected with scrapie (Lee et al., 2005), ERK has also been shown to be involved in the neurotoxic response to PrP 106-126 mediated by glial cells (Combs et al., 1999).

Whilst there was no change in AKT activation in infected cells compared to uninfected, ERK activation was up-regulated in infected cells. In order to investigate this further we inhibited ERK activation in cured and infected cells and examined cell viability. Infected cells treated with the MEK inhibitor showed a decrease in cell viability, suggesting that ERK up-regulation is important in SMB survival as this

was not seen in cured cells. The precise reasons for the protective effect of ERK are not clear. ERK has been demonstrated to be important in protection of cells in response to oxidative stress (Han and Holtzman, 2000). As our laboratory has shown that SMB cells are more resistant to oxidative stress, we hypothesised that ERK activation was protecting against oxidative stress induced by infection, however we found no evidence of this. Studies have shown that ERK up-regulation can be protective against other neurodegenerative diseases, treatment of neurones with Aβ oligomers leads to apoptosis, which can be prevented by Docosahexaenoic acid, in part due to an up-regulation of ERK (Florent et al., 2006), this suggests that ERK inhibition might be a useful therapeutic tool against prion toxicity.

We also investigated whether ERK was activated in F21 cells treated with MnPrP but did not see any increase in ERK activation in cells treated for 24h with MnPrP. Work using tissue from scrapie infected brains has previously shown a down-regulation of ERK pathway genes (Sorensen et al., 2008), however after MnPrP treatment 48h we saw an increase in ERK activation. We hypothesise that, as we have shown there is little further cell death after 48h, the remaining cells may up-regulate ERK as a protective mechanism allowing the cells to survive and that this mechanism is similar to the mechanism in SMB cells.

We finally investigated possible upstream activators of ERK. We investigated 14-3-3, which has been shown to be involved in the regulation of a wide variety of proteins including ERK (Xing et al., 2000) and Bad (Datta et al., 2000). We found that 14-3-3 expression was increased in infected cells and therefore may be one of the upstream proteins involved ERK regulation. 14-3-3 has been hypothesised to prevent apoptosis by a variety of mechanisms including by binding Raf leading to activation of ERK and this is postulated to protect cells. 14-3-3 has been found in the CSF of CJD patients (Hsich et al., 1996) although why it is there remains unclear. It may be due to up-regulation of a variety of signalling mechanisms as 14-3-3 involved in many signalling responses.

Overall our findings have demonstrated that cell death in MnPrP is due to apoptosis, and that this is at least partially caspase 3 mediated, probably in response to p38 activation. We have also demonstrated that there is no evidence of apoptosis in cells

infected with scrapie and that these cells have developed a signalling mechanism involving ERK, which allows them to survive the infection. This up-regulation of ERK activation is also observed in cells that survive MnPrP treatment, suggesting that modulating expression of certain survival signalling pathways could be useful therapeutically. Much more still need to be done to elucidate the pathways involved in the cell death signalling that occurs when cells are treated with MnPrP and the pathways that regulate SMB survival. Elucidation of the upstream pathways may give us a better understanding of the precise causes of neuronal cell death in prion disease and other neurodegenerative diseases.

Further work should look at possible upstream targets of ERK, this could provide a therapeutic target. Work has suggested that microglial cells treated with PrP 106-126 up-regulate ERK via PKC which is activated in response to calcium, although in contrast to our study ERK is not protective but results in the production of neurotoxic signalling molecules (Combs et al., 1999). However PKC activation should be investigated in our model. Work investigating whether MnPrP can activate glial signalling may also give us a better understanding of toxicity of the MnPrP. Further work should also investigate ERK expression *in vivo*, as previous work has shown it is up-regulated in hamsters infected with scrapie (Lee et al., 2005), however the role of ERK over-expression has not been elucidated, investigations using transgenic mice over-expressing ERK could determine the role of ERK in prion diseases.

As well as investigating protective signalling mechanisms elucidation of the pathway(s) involved in MnPrP cell death could provide therapeutic targets. As oxidative stress has been implicated in cell death in prion disease further investigation into the whether MnPrP treatment results in free radical production could provide a clue to the upstream pathways involved in apoptosis. Investigations have shown that PrP 106-126 triggers reactive oxygen species overflow in cells leading to up-regulation of both ERK and JNK resulting in apoptosis (Pietri et al., 2006).

6. Rescuing cells from MnPrP induced cell death

Although there has been intensive study of prion diseases no suitable therapies have been developed that are able to slow or halt disease progression. Having established a model of toxicity using MnPrP our aim in this chapter was to find a method of preventing the toxicity of MnPrP.

PrP^{c} has been shown to have superoxide dismutase activity, leading to the belief that it may be involved in preventing oxidative stress in the brain. Mice lacking the PrP protein have been shown to express higher levels of oxidative stress markers (Wong et al., 2001c). In the disease state, the brains of scrapie infected mice also exhibit oxidative stress even before clinical symptoms develop (Yun et al., 2006). Therefore reducing oxidative stress may prevent cell death in prion disease.

The level of PrP^{c} in the cell has also been shown to effect the incubation time of the disease (Manson et al., 1994; Prusiner et al., 1993). PrP^{c} is also up-regulated in disease (Voigtlander et al., 2001) suggesting that PrP^{sc} may have some effect on the promoter. Therefore down regulating PrP may decrease toxicity. In this regard regulating the prion promoter activity may be of benefit.

The prion protein is encoded by the *prnp* gene which has been well characterised in several species. In mice it consists of three exons and two introns, with the third exon encompassing the entire open reading frame (Westaway et al., 1994). All the prion promoters studied so far lack a TATA box. However various binding sites have been found that enhance and inhibit promoter activity. These include Sp-1 binding sites and AP-1 and AP-2 binding sites, which can inhibit and enhance promoter activity depending on which transcription factors bind to them (Saeki et al., 1996b). Heat shock elements have been found in the rat promoter (Shyu et al., 2002), and heat shock proteins have been shown up-regulate prion protein expression in humans cells (Shyu et al., 2000), there is also an increase in expression of heat shock proteins in disease (Tortosa et al., 2007).

Whilst mammals are susceptible to prion diseases there is no evidence of these diseases in species such as reptiles and birds, although prion protein analogues are found in both species. There is much less conservation of the prion protein sequence between mammals and non-mammalian species. A notable example of the large differences in prion protein structure is the prion protein analogue found in Xenopus. This protein is missing the octarepeat region (Calzolai et al., 2005) involved in binding metals. If non-mammalian PrP retains the function of mammalian PrP and cannot be converted to PrP^{sc} it may provide protection from PrP toxicity.

This part of our study aims to further understand the mechanisms that lead to cell death in the model we have developed, and used a variety of methods to try and prevent cell the cell death observed. Initially we investigated whether MnPrP increased the levels of reactive oxygen species in system, and found that there was a significant increase in levels when cells were treated with MnPrP, however prevention of the increase in ROS using antioxidants was not enough to prevent cell death suggesting that this may not be the main cause of cell death in our system.

We went on to investigate whether over expression of Xenopus or turtle PrP was able to prevent cell death induced by MnPrP. Prion disease does not occur naturally in these species, leading us to hypothesise the prion protein in these species may be resistant to conversion. However over expression of Xenopus PrP was unable to protect cells from MnPrP induced cell death, and turtle PrP was only able to slightly reduce the levels of cell death caused by MnPrP, suggesting that cell death signalling is able to take place regardless of the species of PrP being expressed by the cell.

The prion promoter can be modulated by a variety of factors including neuronal growth factor (Zawlik et al., 2006) and copper (Varela-Nallar et al., 2006), we wanted to investigate whether PrP could modulate the prion promoter. We found that apoPrP, but not MnPrP could modulate the prion promoter.

6.1 Generation of Reactive Oxygen Species.

F21 cells were treated with MnPrP or apoPrP and reactive oxygen species were assessed using a fluorescent probe. The probe, CM-H_2DCFDA, is a cell permeable probe. However once oxidised to it fluoresces and is no longer cell permeable so remains inside the cell. It can be oxidised by hydrogen peroxide, organic hyroperoxides, nitric oxide and peroxynitrite. Cells were treated for 4h prior to the addition of the probe. Fluorescence was measured at time 0, 1 and 2h after the addition of the probe. The MnPrP treated wells had significantly higher levels of ROS when compared to apoPrP (figure 6.1).

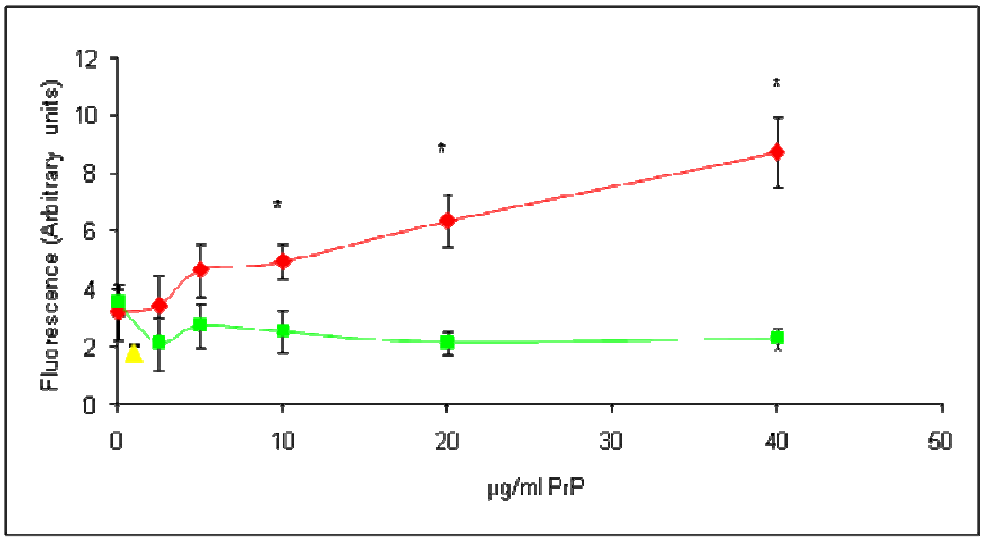


Figure 6.1: Cellular ROS production by F21 cells treated with MnPrP. F21 cells were treated for 4h with varying concentrations of MnPrP (red line), apoPrP (green line) or a manganese control (yellow line). After 4h the media was removed and replaced with the fluorescent probe DCDFDA. Cell fluorescence was then assessed. Error bars are SEM and each point represents at least 6 repeats. Significance was assessed using a one way ANOVA $p<0.01$.

As MnPrP was removed when the probe was added we wanted to investigate whether adding both the probe and the MnPrP the same time would make any difference to the levels of ROS produced. F21 cells were treated with the probe and MnPrP at the same time, this time the cells were not pre-incubated but ROS production was immediately assessed and then assessed again after 2h. An increase in cell ROS production was still observed in cells treated in this manner (data not shown).

The increase in ROS observed in F21 cells treated with MnPrP was also observed in SMB cells treated with MnPrP (figure 6.2), although this required higher concentrations of MnPrP than in the F21 cells. This may be due to the fact that SMB cells cannot be plated as densely however it may be due to SMBs producing less ROS in response to MnPrP.

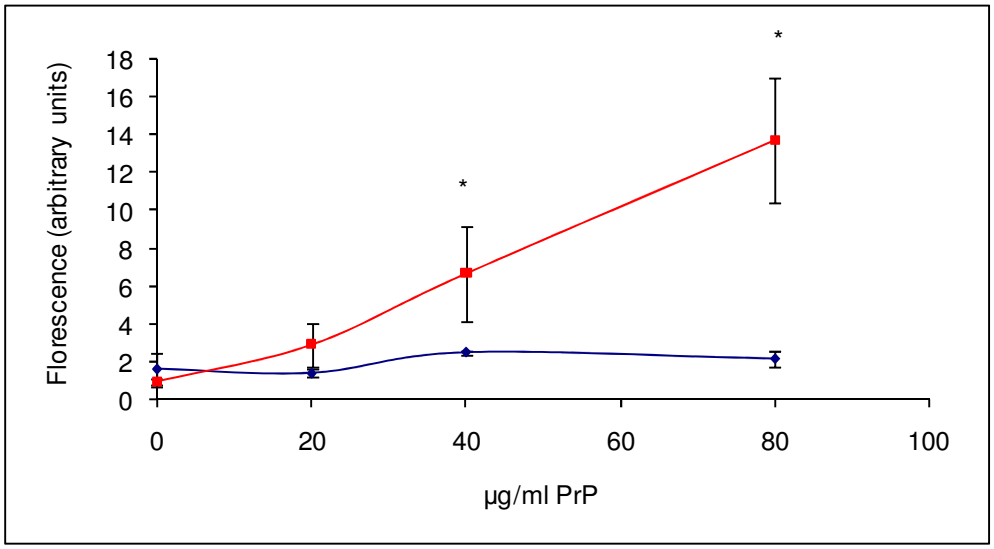


Figure 6.2: Cellular ROS production by SMB cells treated with MnPrP. SMB cells were treated for 4h with varying concentrations of MnPrP (red line), apoPrP (blue line). After 4h the media was removed and replaced with the fluorescent probe DCDFDA. Cell fluorescence was then assessed. Error bars are SEM and each point represents at least 6 repeats. Significance was assessed using a one way ANOVA $p<0.01$.

We went on to investigate whether antioxidants could reduce the levels of ROS induced by MnPrP. We first investigated All-Trans-Retinoic-Acid (ATRA), this is a powerful antioxidant, which has previously been shown to reduce PrP expression (Rybner et al., 2002).

Initial experiments showed that ATRA was able to significantly reduce the levels of ROS generated by treatment with MnPrP (figure 6.3). However further work revealed that ATRA was toxic at levels high enough to significantly reduce ROS expression in MnPrP treated cells, making this an unsuitable antioxidant to use in these experiments. We went on to investigate whether low levels of ATRA could protect, possibly by another mechanism. F21 cells were treated for 24h with varying concentrations of MnPrP and either 0.5µM or 1µM ATRA and viability was assessed

by MTS assay (figure 6.4). No difference was observed in survival suggesting that ATRA is not able to save cells from MnPrP induced apoptosis.

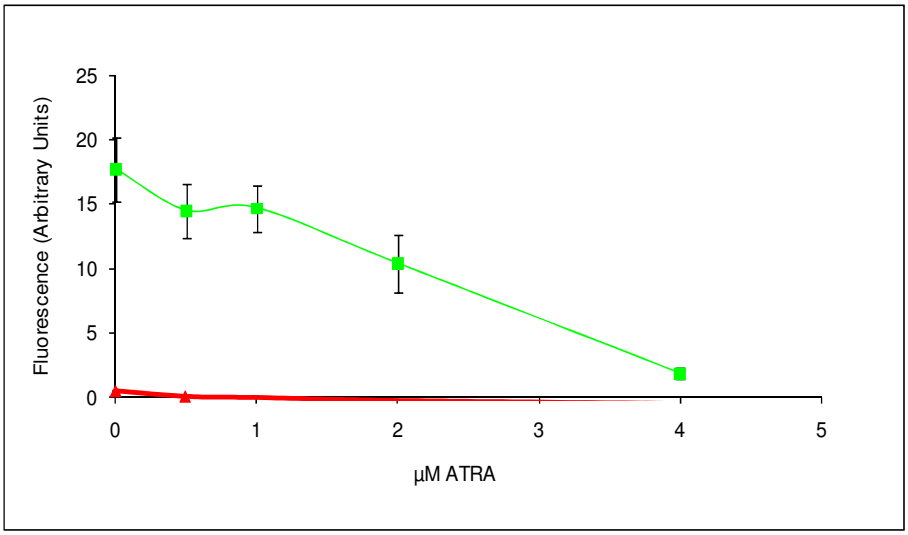


Figure 6.3: The effect of ATRA on cellular ROS expression by F21 cells. F21 cells were treated for 4h with varying concentrations of ATRA and MnPrP. After 4h the media was removed and replaced with the fluorescent probe DCDFDA. Cell fluorescence was then assessed at time 0 (red line) and after 2h (green line). Error bars are SEM and each point represents at least 3 repeats.

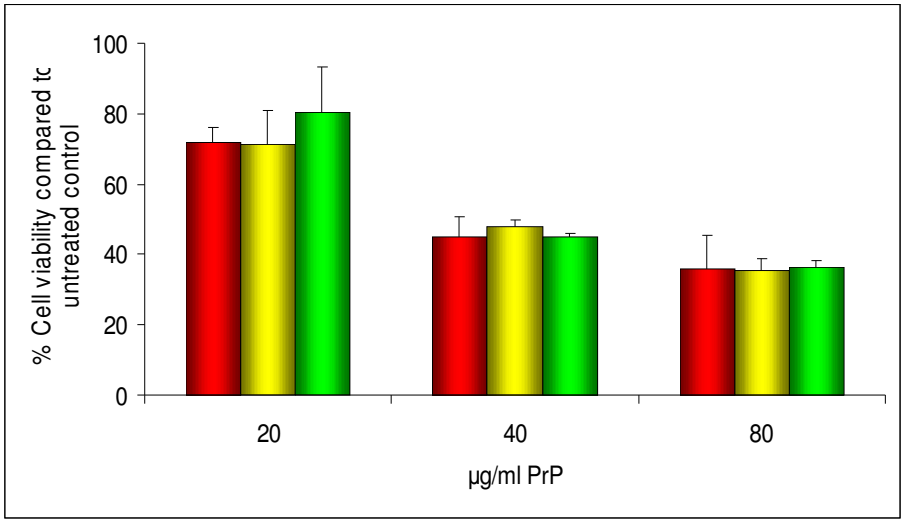


Figure 6.4: Survival of F21 cells treated with MnPrP and ATRA. F21 cells were treated for 24h with varying concentrations of MnPrP alone (red) MnPrP and 0.5µM ATRA (yellow) or MnPrP and 1µM ATRA (green). Cell viability was assessed by MTS assay. Error bars are SEM and each point represents at least 3 repeats.

We used the antioxidants vitamin C (ascorbic acid) and E (α-tocopherol) in order to further investigate whether antioxidants could be useful in rescuing cells treated with

MnPrP. We initially investigated whether either antioxidant was able to reduce basal levels of F21 ROS production. Cells were treated with either 40mM ascorbic acid or 10μM tocopherol and the probe. ROS production was measured after addition of the probe and then at 1 and 2h (figure 6.5).

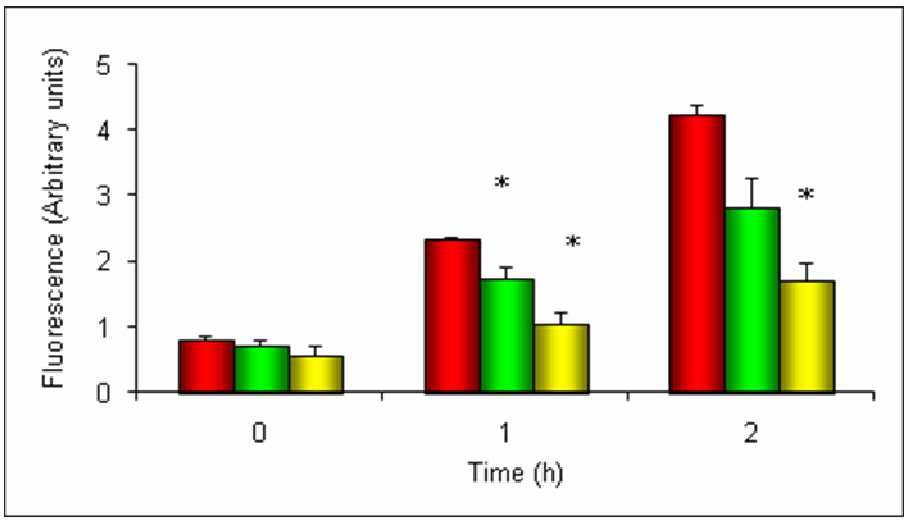


Figure 6.5: The effect of ascorbic acid and α-tocopherol on cellular ROS expression by F21 cells. F21 cells were either untreated (red) or treated with ascorbic acid (green) tocopherol (yellow) and treated with the fluorescent probe DCDFDA. Cell fluorescence was then assessed at time 0 after 1h and after 2h. Error bars are SEM and each point represents at least 3 repeats. Significance was assessed using a Student's T-Test $p<0.05$.

Tocopherol was able to significantly reduce basal levels of ROS production when compared to the control. Ascorbic acid was less effective and was only able to significantly reduce ROS production at 1h.

We then investigated the ability of both ascorbic acid and tocopherol to reduce the levels of ROS produced by MnPrP treatment. Cells were either pretreated with the antioxidants for 4h and then the probe and MnPrP were added or cells were treated with probe, antioxidant and MnPrP at the same time. Both ascorbic acid and tocopherol were able to significantly reduce the level of ROS produced by MnPrP treated cells. The reduction greater if the cells were not pretreated, but added with the MnPrP (figure 6.6). We ensured the levels of both antioxidants did not affect cell viability after 24h of treatment (figure 6.7).

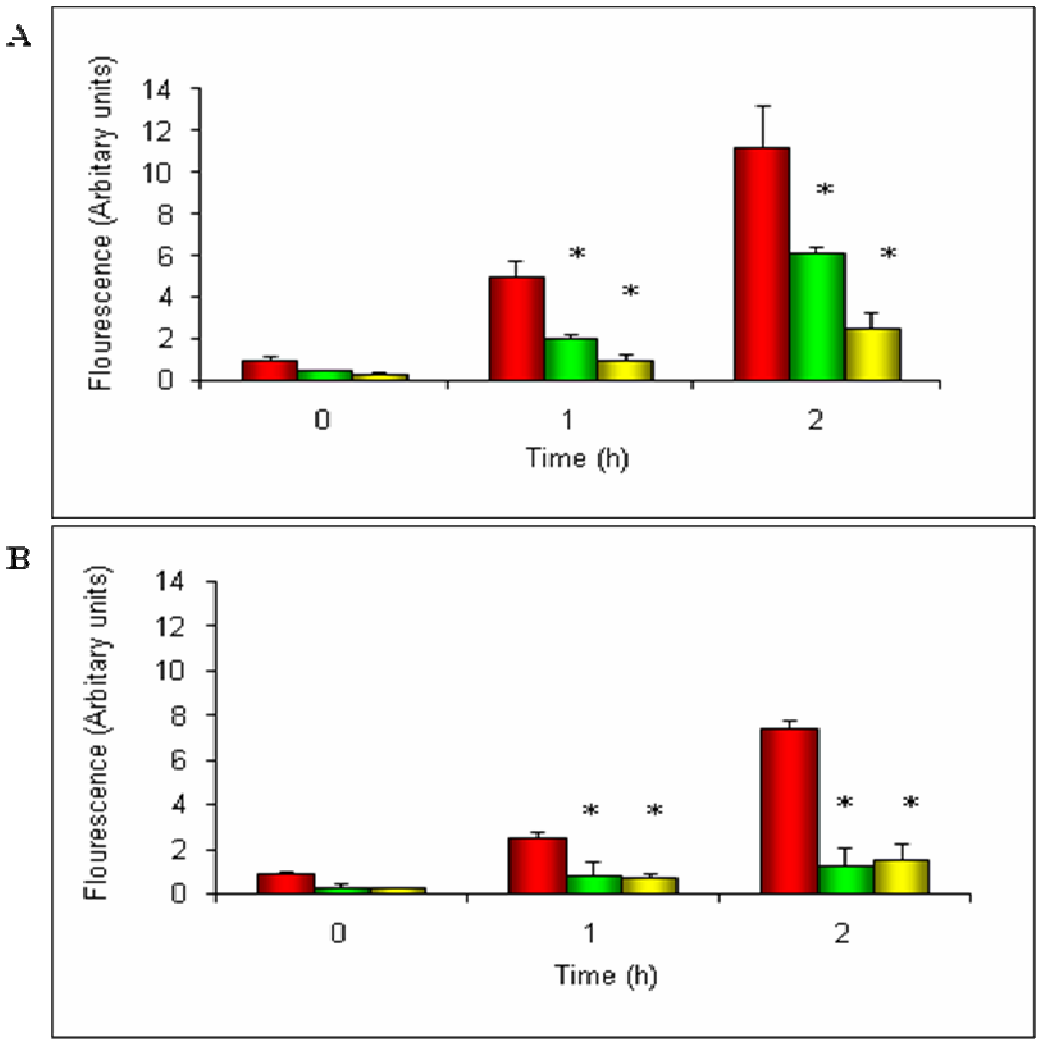


Figure 6.6: Effect of ascorbic acid and tocopherol on ROS production by F21 cells treated with MnPrP. A: F21 cells were pretreated with either ascorbic acid (green) or tocopherol (yellow) or untreated (red). Cells were incubated for 4h prior to the addition of the probe and 40µg/ml MnPrP. Fluorescence was then assessed. B: F21 cells were treated with either ascorbic acid (green) or tocopherol (yellow) or untreated (red), prior to the addition of the probe and 40µg/ml MnPrP. Fluorescence was then assessed. Error bars are SEM and each bar represents at least 3 repeats. Significance was assessed using a Student's T-Test *<0.05.

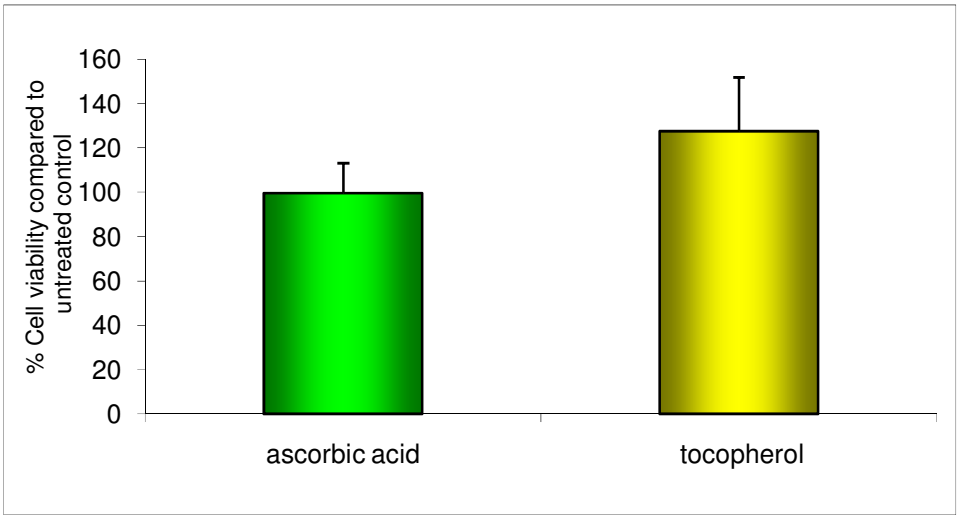


Figure 6.7: Effect of ascorbic acid and tocopherol on F21 cell survival. F21 cells were treated for 24h with either 40mM ascorbic acid or 10µM tocopherol for 24h and viability was assessed using an MTS assay. Error bars are SEM n=3. There was no significant difference between the control and either ascorbic acid or α-tocopherol as assessed by students T-test.

F21 cells were treated with both MnPrP and the antioxidants and cells were incubated for 24h. Cell viability was assessed using an MTS assay. No difference was seen in viability whether cells had been treated with the antioxidant or not (figure 6.8).

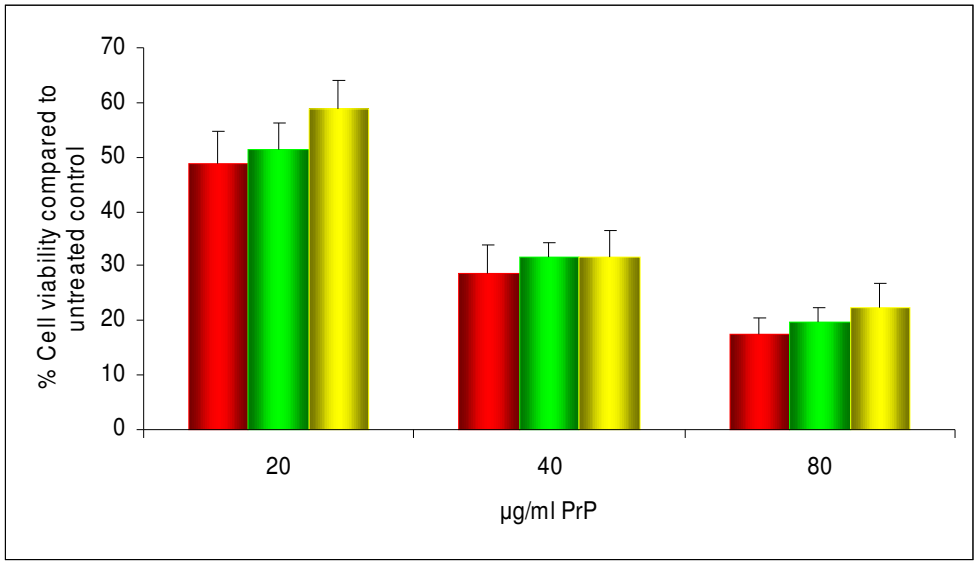


Figure 6.8: Effect of ascorbic acid and tocopherol on MnPrP treated F21 cell survival. F21 cells were treated with either MnPrP alone (red), ascorbic acid and MnPrP (green) or tocopherol and MnPrP (yellow) for 24h. Viability was assessed using a MTS assay, no points were significant as assessed by Students T-test.

We hypothesised that the antioxidants used might not survive for long periods of time at 37°C and so we investigated whether there was any difference in viability of cells treated with antioxidants and MnPrP for a shorter time period of 8h (figure 6.9). There was no difference in viability, indicating that despite being able to significantly reduce the levels of ROS produced by cells treated with MnPrP antioxidants are not sufficient to reduce MnPrP toxicity.

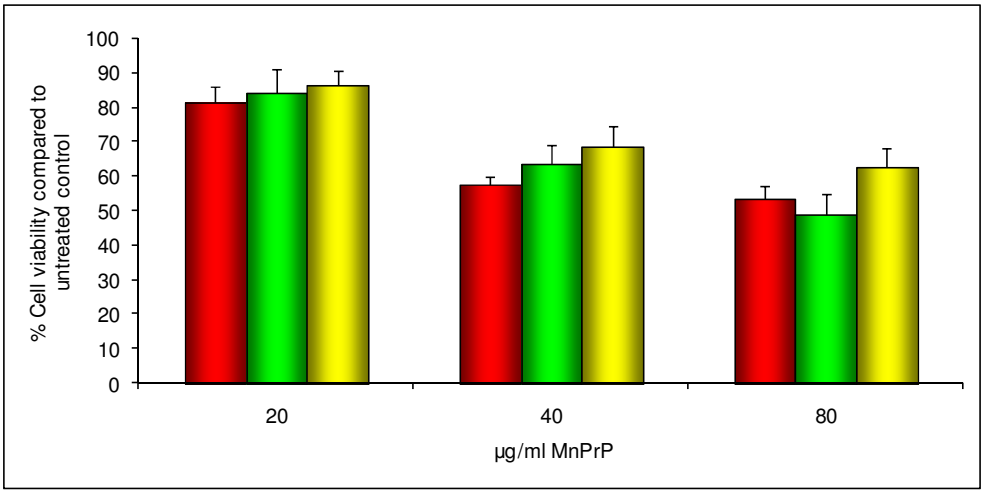


Figure 6.9: Effect of ascorbic acid and tocopherol on MnPrP treated F21 cell survival. F21 cells were treated with either MnPrP alone (red), ascorbic acid and MnPrP (green) or tocopherol and MnPrP (yellow) for 8h. Viability was assessed using a MTS assay, no points were significant as assessed by Students T-test.

6.2 The Prion Promoter

Cellular PrP expression is required for disease pathogenesis and the level of PrP^c expression has been shown effect disease incubation (Manson et al., 1994; Prusiner et al., 1993). Therefore the modulation of expression of PrP by the promoter may alter disease progression.

F14 cells, lacking PrP^c expression, were transiently transfected with either the full-length mouse promoter which had been tagged with GFP or with a GFP tagged CMV promoter both were in the pd2-EGFP vector and had been created as previously described (Haigh et al., 2007). The CMV (Cytomegalovirus) promoter is constitutively expressed and acts as a control (figure 6.10).

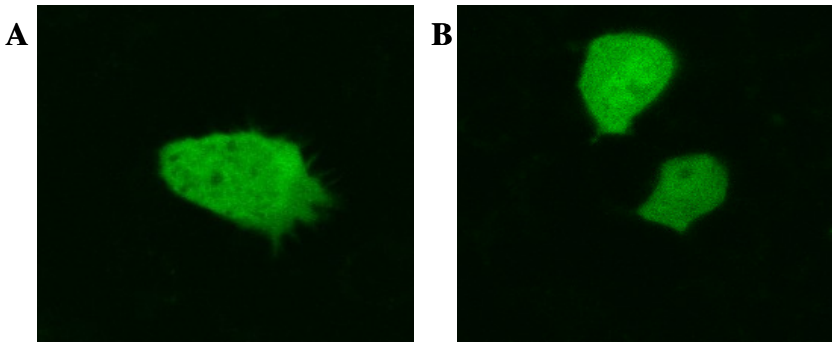
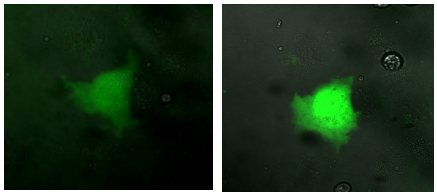
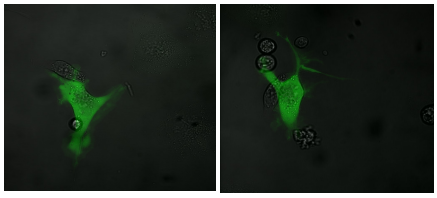


Figure 6.10: F14 cells transfected with the mouse PrP promoter or CMV promoter. Cells were transiently transfected with A: A WT mouse prion promoter tagged with GFP B: A CMV promoter tagged with GFP. Expression was assessed using confocal microscopy.

Previous work has shown that GFP expression was up-regulated by DMSO in F14 cells expressing the full length promoter suggesting DMSO was able to modulate the promoter. In order ensure that the promoter could be modulated transfected cells were treated for 1h with 1% DMSO and there was a visible increase in GFP expression (figure 6.11).



WT cells after 1hr incubation with 1% DMSO



CMV cells after 1hr incubation with 1% DMSO

Figure 6.11: The effect of DMSO on promoter expression. Incubation of WT and CMV cells with 1% DMSO for 1h. Images were captured using a confocal microscope at 63x magnification.

To investigate the ability of MnPrP and apoPrP to modulate the expression of the promoter we treated cells for 4h with 80 µg/ml PrP or with a manganese control and GFP intensity was assessed. Interestingly the apoPrP was able to significantly down-regulate PrP promoter expression, whilst MnPrP had lost this ability (figure 6.12). Therefore the binding of manganese to the prion protein inhibits its ability to

regulate the promoter. There was no difference in intensity in CMV promoter expression with any treatment.

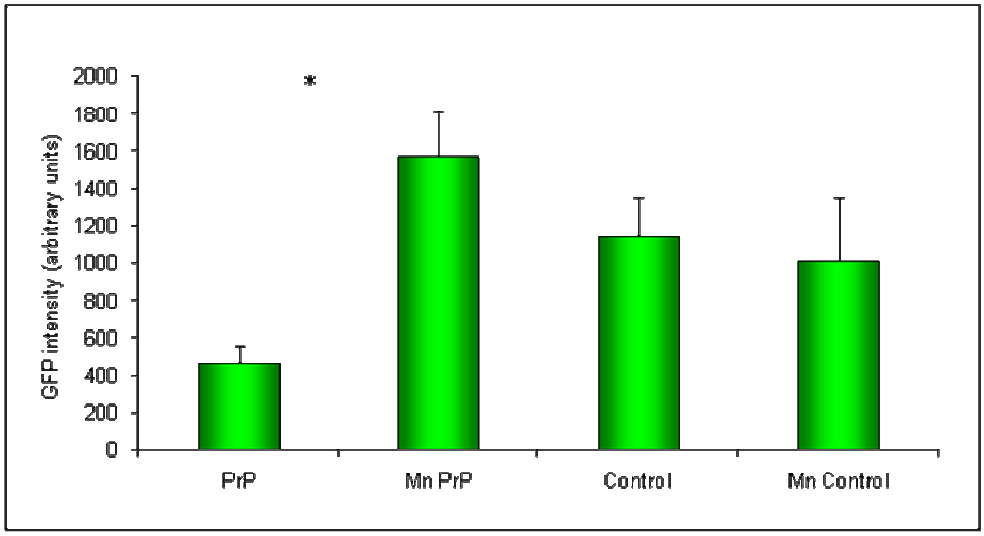


Figure 6.12: The effect of MnPrP on promoter expression. Cells were transfected with WT promoter and treated for 4h with either apoPrP, MnPrP or a Manganese control. Cellular GFP intensity was assessed using Zeiss LSM software. Each bar represents at least 25 cells. Error bars are SEM. Significant points were assessed using students T-test *p<0.05.

6.3 Non Mammalian Prion Proteins

The prion protein is highly conserved between mammals; however PrP analogues found in non-mammalian species are less well conserved. Evolutionary speaking Xenopus PrP is one of the most distant prion proteins to be fully characterised and is completely missing the octarepeat region (Calzolai et al., 2005) (figure 6.13). We wanted investigate if Xenopus prion protein could protect cells from MnPrP toxicity as prion expression has been shown to be required for toxicity. We hypothesised as Xenopus do not naturally develop prion disease the PrP they express may not be susceptible to conversion and therefore could protect against prion toxicity. Cells were stably transfected with either GFP tagged mouse PrP or GFP tagged Xenopus PrP. Expression was checked visually prior to any experiment. Previous work using confocal microscopy had shown that constructs were expressed at the cell surface, as would be expected (figure 6.13), In order to express turtle and Xenopus in a mammalian cells parts of the mouse PrP sequence were used, meaning the protein was correctly processed to the cell surface. The mouse construct contained the GFP sequence inserted at amino acid 42, the full mouse sequence is included in it. The Xenopus and turtle constructs contained the mouse sequence from amino acid 1 to

42, then the GFP and then the turtle or Xenopus sequence, the mouse sequence was also included at the C-terminus end of the protein from amino acid 231, again to allow correct processing of the protein. The construct was inserted into pEGFP-C1 vector (figure 6.13).

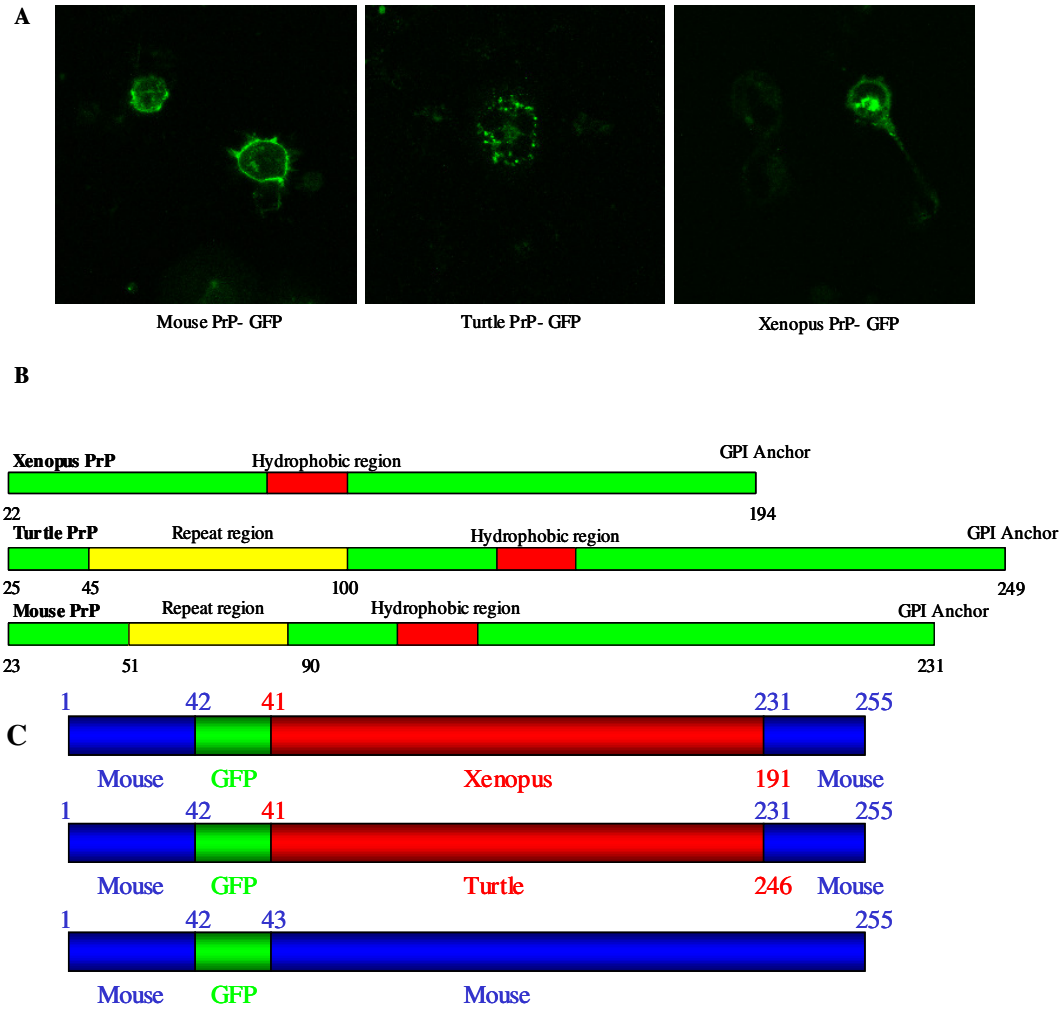


Figure 6.13: Expression of non-mammalian prion proteins. A: Confocal microscopy of F14 cells transfected with mouse, turtle or Xenopus PrP tagged with GFP (Pictures kindly donated by Dr Cathryn Haigh). B: A schematic diagram of Xenopus mouse and turtle PrP. C: A schematic diagram showing the GFP construct used, mouse regions were used to ensure the protein was correctly expressed.

Cells were plated and treated for 24h with varying concentrations of PrP and cell viability was assessed using an MTS assay. There was no difference in the viability

of cells transfected with Xenopus PrP or mouse PrP suggesting that Xenopus PrP is not able to protect cells from MnPrP induced toxicity (figure 6.14).

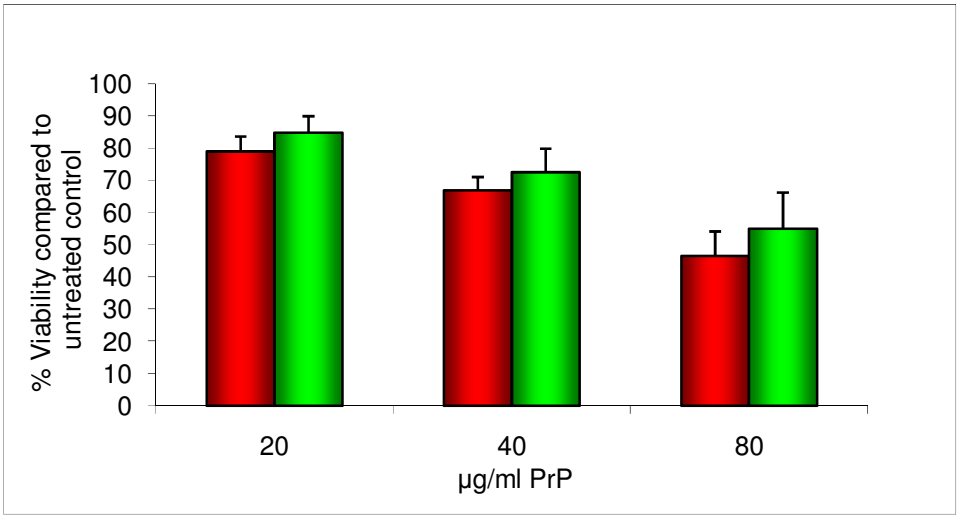


Figure 6.14: Effect of Xenopus PrP expression on cell survival in response to MnPrP. Cells were transfected with a GFP tagged mouse PrP construct (red) or a GFP tagged Xenopus PrP construct (green). Transfected cells were then treated for 24h with varying concentrations of MnPrP. Viability was assessed by MTS assay. Error bars are SEM, n=9. No points were significant as assessed by students T-Test.

We also investigated the ability of the expression of turtle PrP to protect the cells from MnPrP toxicity. Turtles do not develop prion disease naturally like Xenopus, but unlike Xenopus they retain a repeat region although it is a hexarepeat rather than an octarepeat. F14 cells were transfected with either a GFP tagged mouse PrP construct or a GFP tagged turtle PrP construct, which was expressed at the cell surface (figure 3.13). Cells were then treated for 24h with MnPrP for 24h. Interestingly cells expressing turtle PrP showed a slight but significant increase in cell viability when compared to cells expressing mouse PrP suggesting that turtle PrP may be able to protect cells from MnPrP toxicity (figure 3.15).

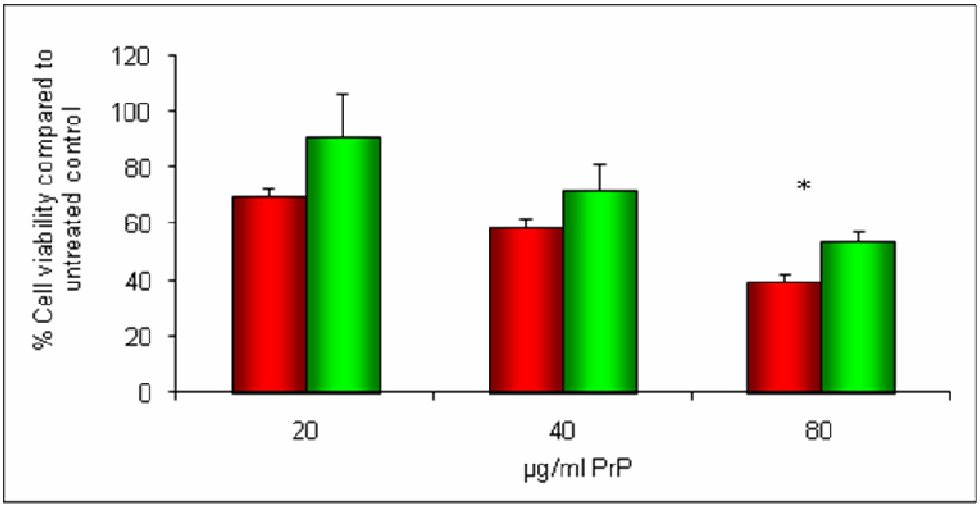


Figure 6.15: Effect of turtle PrP expression on cell survival in response to MnPrP. Cells were transfected with a GFP tagged mouse PrP construct (red) or a GFP tagged turtle PrP construct (green). Transfected cells were then treated for 24h with varying concentrations of MnPrP. Viability was assessed by MTS assay. Error bars are SEM, n=3. *<0.05 as assessed by students T-Test.

6.4 Discussion

A large body of work has suggested a link between prion disease and oxidative stress. In the normal state the prion protein has been demonstrated to be important in preventing oxidative stress (Brown et al., 1997b; Watt et al., 2007; Wong et al., 2001c). In the disease state there is evidence of markers of oxidative damage (Freixes et al., 2006; Guentchev et al., 2000; Yun et al., 2006), although it is not clear whether this is due to the loss of the normal prion protein or the disease process.

Our study has revealed that MnPrP increases the amount of ROS produced by F21 cells whilst apoPrP does not increase ROS production. Previous studies using other models of prion toxicity, have also shown an increase in oxidative stress. Cells treated with toxic levels of PrP 106-126 have increased heme-oxygenase (HO-1) expression, a marker of oxidative stress (Rizzardini et al., 1997). Microglial cells have been shown to release nitric oxide in response to PrP 90-231 (Thellung et al., 2007). As in our model studies have suggested the importance of metals in the generation of ROS by the prion protein. A C-terminal fragment of PrP containing several known disease related mutations was found to generate hydrogen peroxide in the presence of iron and this was hypothesised to result in enhanced toxicity of the protein (Turnbull et al., 2003b).

We hypothesise that MnPrP, like PrP^{sc}, is able to induce oxidative-stress and this requires the presence of metals. In order to inhibit the production of ROS by cells treated with MnPrP we treated the cells with the antioxidants tocopherol or ascorbic acid. Tocopherol has been shown to protect neural tissue form damage by oxidative stress *in vitro*. Studies showed that rat neurons subjected to hypoxia could be saved by tocopherol treatment (Tagami et al., 1998). Ascorbic acid has been shown to reduce neuronal apoptosis observed in the developing brains of rats exposed to arsenic, which causes oxidative stress (Tagami et al., 1998). However there has been little research into specific antioxidant therapies in CJD, although α--tocopherol has also been shown to reduce the toxicity of PrP 106-126 in cerebellar cultures (Brown et al., 1996) and a single case of treatment with antioxidants has been reported, with

an increase in life expectancy (Drisko, 2002). There has been much more research into the use of antioxidants in Alzheimer's disease as a possible therapy, although α--tocopherol, has been shown to be fairly ineffective (Boothby and Doering, 2005).

In our model whilst antioxidant treatment was able to reduce the levels of ROS produced by MnPrP, this did not rescue the cells from MnPrP induced apoptosis. This may be due to the fact that we did not use high enough concentrations of antioxidants. Previous studies using tocopherol to inhibit prion induced cell death have used much higher concentrations (Brown et al., 1996). However in our system concentrations higher than used resulted in an increased in cellular proliferation so could not be used in conjunction with the cellular proliferation assay used to assess cell death. As the levels used resulted in a significant reduction in ROS levels it could be suggested that ROS may not be the major component in cell death in our system. However *in vivo* oxidative stress may be relevant especially as prion diseases are more prevalent in the elderly who are more susceptible to oxidative stress (Urano et al., 1998).

One of the limitations of the work is the use of a general probe for ROS rather than using more specific probes. Further investigation of the reactive oxygen species produced when cells are treated with MnPrP may provide more insight whether there is a specific reactive oxygen species produced, and allow us to elucidate the signalling pathway that leads to it production. Previous studies have highlighted some pathways that maybe involved in ROS production in prion disease. The 106-126 peptide can increase ROS production in a neuronal cell line and it is suggested this is due to an interaction with PrP^c on the cell surface involving the signalling molecule fyn-kinase, a molecule widely thought to be involved in PrP^c signalling, and this ROS production could be reduced using a fyn-kinase inhibitor (Pietri et al., 2006). Other studies have suggested other pathways that maybe involved in ROS production in prion disease, treatment with 106-126 has been shown to increase cystolic Ca^{2+} levels leading to an increase in the production of ROS which could be reduced by preventing Ca^{2+} release from the ER (Ferreiro et al., 2008b; Ferreiro et al., 2006). Future work should concentrate on investigating the types of ROS produced in our model and the signalling mechanisms involved, this would provide a better understanding of the role of ROS in our model.

The increase in ROS observed in F21 cells treated with MnPrP was also observed in SMB cells treated with MnPrP. This is perhaps unsurprising as MnPrP is also toxic to SMB cells. However the dose of MnPrP required to induce ROS production was higher than in the F21 cells. This may be due to the fact that SMB cells cannot be plated as densely. This would need to be investigated, either by ensuring that identical numbers of cells were plated or using a different method such as flow cytometry which can count individual cells. If the difference is not due to a disparity in plating, the differences observed could also be due to changes in the signalling pathways involved in ROS induction. These signalling pathways may not being activated in response to low levels MnPrP in SMB cells as readily as F21 cells. This could be protective response developed by SMB cells to resist PrP^{sc} toxicity. Initial observations in our laboratory have shown that SMB cells are more resistant oxidative stress than SMB-PS cells. Further work could investigate whether SMB and SMB-PS cells require different concentrations of MnPrP to induce ROS production.

Prion expression has been shown to be important in disease pathogenesis. Down-regulation of PrP expression via the prion promoter may be able to increase or decrease cell death observed. The promoter can be modulated by several factors, including copper. From a therapeutic point of view perhaps the most interesting factor is all-Trans retinoic acid (ATRA). ATRA is a vitamin A derivative already used in the treatment of promyelocytic leukaemia. It has been shown to down-regulate PrP expression although the mechanisms by which it does this are unclear but could involve the transcription factor AP-2 (Rybner et al., 2002). AP-2 has been suggested to exert an effect on the *prnp* gene (Cabral et al., 2002).

As previous studies had shown that PrP expression was up-regulated in prion disease (Voigtlander et al., 2001) we initially hypothesised that PrP^{sc} might be up-regulating the expression of PrP by acting on its own promoter. In order to test this in our model we investigated whether MnPrP could up-regulate the PrP promoter leading to an over-expression of PrP^{c}.

In vivo if PrP^{sc} was able to regulate its own promoter, thereby increasing prion protein expression, this would lead to a decrease in incubation time, as over-

expression of PrP in mice has been shown to lead to a shorter incubation time (Bueler et al., 1993; Manson et al., 1994). We found that MnPrP showed a tendency to increase promoter activity, although this was not significant. ApoPrP was able to down-regulate PrP expression. The ability of PrP to regulate its own promoter would be extremely important in PrP toxicity and *in vivo* in disease progression. We hypothesise that the down-regulation observed when cells are treated with apoPrP, would lead to a decrease in PrP expression and a correspondent decrease in the toxicity, as PrP expression is required for PrP toxicity (Novitskaya et al., 2006). Conversely the tendency of MnPrP to up-regulate PrP promoter activity would result in an increase in PrP expression and a resulting increase in toxicity. ApoPrP may be able to regulate the prion promoter as once in the media it may bind copper giving a structure like PrP^c, it may then be able to modulate signalling leading to a decrease in PrP expression. This self-regulation of PrP keeps the cellular level of PrP low. The up-regulation of the promoter by MnPrP may be one of the causes of MnPrP toxicity. In order to investigate whether MnPrP does up-regulate PrP expression and if apoPrP can down-regulate PrP expression levels of PrP expression should be assessed at the protein level using Western blotting. This would allow us to confirm that the increase or decrease in promoter activity was functional rather than an artefact. This would then allow us to investigate how PrP is able to regulate its own promoter. PrP may interact directly with promoter and the change in response may be due to the change in protein structure and is ability to directly interact with the promoter. If PrP is not able to enter the nucleus and interact directly with the prion promoter change in expression may come either from apoPrP interacting directly with a cell surface receptor or PrP^c on the cell surface or indirect activation of signalling pathways. The prion promoter has been shown to associate with several cellular proteins including laminin (Gauczynski et al., 2001) and fyn kinase (Pietri et al., 2006), however there is no evidence that PrP binding to receptors can result in a change in promoter activity.

As oxidative stress has been shown to be regulate the prion promoter (Shyu et al., 2004), it maybe the increase in ROS produced by MnPrP that leads to the up-regulation of the PrP promoter. Studies have also shown that the signalling molecule MEK is able to activate the PrP promoter in response to neuronal growth factor

(NGF) (Zawlik et al., 2006). As MEK is activated by MnPrP (via ERK) in our system this may be the reason for the increase in promoter activation observed.

There has been extensive work investigating the species barrier that generally prevents the spread of prion disease between different species. Studies have demonstrated that inoculation of mice with hamster scrapie generally does not result in disease, unless the mice express HaPrP (hamster PrP) (Prusiner et al., 1990; Scott et al., 1989). Further work demonstrated that mice expressing HaPrP specifically in astrocytes were susceptible to prion disease. However expression of mouse PrP prevented this susceptibility (Raeber et al., 1997). This suggests that expression of PrP may have an inhibitory effect on the conversion of PrP from other species. Although there are notable exceptions to this barrier, including the proposed spread of BSE into the human population as vCJD (Bruce et al., 1997). We hypothesised that by expressing non-mammalian prion proteins we could prevent the toxicity of MnPrP. We choose to use non-mammalian prion proteins as they show little sequence homology to mouse PrP, although they do retain a surprisingly similar secondary structure (Ahn and Son, 2007; Calzolai et al., 2005). Xenopus PrP does not have the octarepeat region found in most prion proteins (Calzolai et al., 2005) and unpublished data from our laboratory has shown that it does not bind metals. A chimera of Xenopus and mouse PrP expressed in cells has shown that the N-terminal region of Xenopus, despite missing the octarepeat region, is able to direct subcellular trafficking of the prion protein properly suggesting that the N-terminus is required as targeting signal for subcellular trafficking (Nunziante et al., 2003). This also suggests that Xenopus/mouse chimera can be successfully expressed in mammalian cells, this confirms our observations that the chimera appeared to be properly expressed at the cell surface. Turtle PrP is closer in primary structure to mouse PrP, still retaining some of the metal binding motifs (Calzolai et al., 2005). Neither turtle PrP nor Xenopus PrP expression was able to completely save cells from MnPrP induced cell death, although turtle PrP expression did give a slight protective effect.

In order for the turtle and Xenopus prion protein to be properly processed and expressed in mammalian cells the regions involved in the processing of the protein however these signalling regions should be cleaved during processing and are unlikely to have impact on the ability of the protein to protect the F14 cells from

MnPrP toxicity. However a small part of the N-terminus of mouse PrP would still be present in the protein after processing and the effect of this on toxicity should be investigated.

Previous work using recombinant protein has demonstrated that a human PrP^{res} seed is unable to convert recombinant mouse PrP to PrP^{res} *in vitro* suggesting that the species barrier still exists between recombinant proteins (Baskakov, 2004). This led us to the conclusion that the non-mammalian proteins expressed by the cells must still have the regions required for toxicity, this is perhaps unsurprising given that certain regions have been shown to retain structural motifs that may be important in function. Recent work has suggested that the octarepeat region may be important for conversion (Sakudo et al., 2008), however in our system, expressing Xenopus PrP which does not have the octarepeat region we observed no reduction in prion toxicity suggesting it is not required for prion toxicity. This is also confirmed by work using construct such as the mini-prion which do not contain the octarepeat region but are still toxic to cells (Bonetto et al., 2002).

Further investigation of the ability of turtle PrP to protect against MnPrP toxicity is required, including investigating the effect of the mouse regions in the protein on the ability to protect against toxicity. In order to elucidate whether turtle PrP is able to protect against infection as well as toxicity attempts to infect permissible cell lines expressing turtle PrP should be undertaken. The ability of other non-mammalian prion proteins, such as avian prion proteins, to protect against MnPrP toxicity should also be investigated.

7. General Discussion

7.1 Models of Prion Toxicity

Prion diseases have been studied extensively and multiple models of prion toxicity have been developed. Mouse models have been used to establish the requirement of PrP^c expression for both infectivity and toxicity (Brandner et al., 1996; Bueler et al., 1993; Bueler et al., 1994). Several different animal models have also highlighted the role of neuronal apoptosis in the pathogenesis of prion disease (Fairbairn et al., 1994; Giese et al., 1995). Work using mouse models has revealed deletion of certain regions of the prion protein can result in neurodegeneration in mice suggesting these regions of the protein interact to prevent exposure of highly toxic domains. For example deletion of amino acids 105-125 of the prion protein has been shown to cause neonatal lethality in mice (Li et al., 2007b). A prion protein expressed in the cytosol is also toxic mice (Norstrom et al., 2007). Mouse models provide an accurate model of prion disease, although some, such as the deletion mutants, do not necessarily mimic a situation that would occur naturally in disease but highlight regions important for prion protein stability.

Perhaps the most widely used models of PrP toxicity are cells (either primary or cell lines) treated with either peptides or recombinant PrP. These models provide information on the regions of PrP required for toxicity as well as the mechanism of toxicity. Unlike mouse models they provide a low cost, high throughput model. However the biological relevance of some of the peptides and PrP fragments has been called into question. The most widely used peptide is the 106-126 peptide. This peptide has many of the properties of the prion protein (Forloni et al., 1993) and has been widely used to investigate the mechanism of toxicity of the prion protein (Corsaro et al., 2003; Dupiereux et al., 2006; Ettaiche et al., 2000; Pietri et al., 2006). However it is not a fragment of the prion protein that has been found *in vivo* and as such it cannot replicate the situation *in vivo*. Several other peptides have been found that replicate some properties of the prion peptide. A peptide spanning the putative transmembrane region of the prion protein (amino acids 118-135) has been shown to be toxic to neuronal cells although this was not dependant on cellular PrP

expression, so it does not replicate the *in vivo* situation (Chabry et al., 2003). A peptide that corresponds to a fragment of the prion protein found in the brains of patients of GSS, the fragment has been shown to be toxic to primary cortical neurons and toxicity is dependent on cellular PrP expression (Fioriti et al., 2007). Whilst peptides can be used as model of prion disease, they cannot replicate the differing fragments of PrP found *in vivo* and in the disease, and as such represent a limited model of toxicity.

Many studies have used recombinant protein to investigate the toxicity of the prion protein, however conversion of the protein is often uses chemicals that would not be found at the concentrations used *in vivo* (Post et al., 2000), or thermal denaturation (Corsaro et al., 2006) or shaking (Novitskaya et al., 2006). However recombinant protein has been shown to be selectively toxic to cells that express PrP^{c} (Novitskaya et al., 2006; Post et al., 2000), suggesting that whilst the conversion methods currently used may not be relevant *in vivo* the use of recombinant protein provides a useful model of prion disease. Our model of prion disease uses full-length recombinant PrP which is bound to manganese unlike the conversions described previously there is evidence that manganese is bound to PrP^{sc} *in vivo* (Wong et al., 2001b) so it does represent a valid model of PrP^{sc} *in vivo.*

We showed that MnPrP was significantly more toxic to cell lines than apoPrP and that toxicity of the protein was dependant on cellular PrP expression, representing the situation *in vivo.* However the dose of MnPrP required for toxicity was quite high, other studies using recombinant protein generally used slightly lower doses, 0.5µM was sufficient to cause up to 30% cell death in N2a cells treated with recombinant PrP fibrils (Novitskaya et al., 2006).

Although apoPrP was significantly less toxic than MnPrP it was toxic to cells, this is in contrast to studies using PrP 90-231 which have shown that in its α-helical state is not toxic to cells, although in a β-sheet rich conformation it is highly toxic (Corsaro et al., 2006). The structure of the prion protein is thought to be important for toxicity and previous work using MnPrP has shown that it is partially protease resistant and has a higher β-sheet content once aged, this mimics some of the properties of PrP^{sc}

(Brown et al., 2000) and therefore it is likely the increased β-sheet structure contributed to the toxicity of MnPrP.

In order to investigate the toxicity of MnPrP we choose to use an *in vitro* model, we choose this model for several reasons. Firstly time constraints meant that mouse models would have limited our ability to fully research the toxicity of MnPrP. We were also aware that whilst animal models provide the best replication of disease, cell models can provide greater detail at a cellular level and therefore give us a greater understanding of the underlying causes of MnPrP toxicity. However the lack of an *in vivo* study is one of the greatest limitations of the model and, as misfolded PrP produced *in vitro* has been shown to infect mice (Castilla et al., 2005; Weber et al., 2007), the ability of MnPrP to infect cells and mice should be investigated.

The use of recombinant protein also means we are unable to replicate the glycosylation of PrP. Glycosylation is thought to be important in determining the outcome of prion infection. Mice can sustain infection even when expressing an unglycosylated prion protein, however disease susceptibility is reduced and some strains do require glycosylation for infection suggesting that glycosylation plays an important in prion diseases (Tuzi et al., 2008). Work in an *in vivo* model should establish whether glycosylation is important in toxicity in our model.

There are several strong lines of evidence to suggest that manganese binding to PrP may have a role in prion disease. Manganese has been shown to bind to PrP, with a lower affinity than copper (Brazier et al., 2008; Jackson et al., 2001). The lower affinity for manganese led to a hypothesis that manganese binding would not occur *in vivo*. However recent studies have suggested that the dissociation constant of PrP for manganese is similar to that of other, known, manganese binding proteins (Brazier et al., 2008). They also revealed that manganese binding to PrP did not require copper displacement, which had previously been thought to be a requirement for manganese binding to PrP (Brazier et al., 2008). This strongly suggests that manganese binding to the prion protein could occur *in vivo*. Previous work had also shown that PrP bound to manganese had many of the properties associated with PrP^{sc}, including partial protease resistance and an increase in β-sheet structure. MnPrP had also been shown to be toxic to neuronal cells and this was dependent on

cellular PrP expression (Brown et al., 2000). Increasing evidence has also pointed to a role for manganese in the disease *in vivo*, there is an increase in manganese in the CNS of patients suffering from CJD (Hesketh et al., 2008) and in scrapie infected mice (Thackray et al., 2002) and sheep (Hesketh et al., 2007). Work has also shown that manganese is associated with PrP^{sc} extracted from the brains of patients with sCJD, suggesting the increase in manganese observed in the disease may be due to manganese binding to the prion protein (Wong et al., 2001b). Whilst there is increasing evidence of the relevance of manganese in prion disease the toxicity of manganese bound PrP has not been fully studied, this thesis has revealed that manganese binding results in increased toxicity. We further investigated the role of manganese binding in prion toxicity and demonstrated that if the histidines known to bind manganese were mutated the toxicity of the protein was significantly reduced. This suggested that binding of manganese, to known metal binding sites, was required for toxicity. We have also shown that removal of the octarepeat region increases the toxicity of MnPrP, suggesting that this region has a protective effect. In contrast removal of the fifth site has no effect on toxicity.

Further work could look at the effect of other metals on PrP toxicity, iron has been implicated in the conversion of PrP *in vitro* (Basu et al., 2007) and therefore may have an effect on the toxicity of the prion protein. Work should also investigate the effect of copper binding on PrP toxicity. As copper is thought to be bound to PrP^{c} *in vivo* it would be expected to be less toxic than PrP bound to other metals but there is evidence that copper can cause toxicity, copper has been shown to increase the aggregation of PrP 106-126 and its toxicity (Jobling et al., 2001). This suggests there may be a more complex relationship that needs to be investigated.

There is increasing evidence that metal imbalance maybe relevant in other neurodegenerative diseases suggesting that our model of neurotoxicity could be useful to research into other neurodegenerative diseases. Previous studies have shown that Amyloid Beta (AB), the main constituent of amyloid plaques in the brains of AD patients, can bind copper and zinc (Talmard et al., 2007; Tougu et al., 2008). Copper binding to AB 25-35 has been shown to promote conversion to a more β-sheet structured peptide that is more toxic *in vitro* (Giuffrida et al., 2007). It has also been suggested that zinc binding to AB can cause aggregation (Tougu et al.,

2008). Whilst we have developed a limited understanding of the regions involved in MnPrP toxicity further structural and aggregation studies of the mutants may reveal the reasons for the differing toxicities of mutants and may reveal a similar mechanism of aggregation and toxicity to that of Aβ bound to metals, this may mean that similar therapies are useful in both diseases.

7.2 Cell signalling in prion disease

Prion diseases result in massive neuronal cell death, several studies have shown this is due to apoptosis of the neurones (Fairbairn et al., 1994; Giese et al., 1995; Gray et al., 1999). However the signalling mechanisms that initiate apoptosis remain unclear. Several studies have shown that caspase 3 cleavage is involved in initiating apoptosis in prion disease (Corsaro et al., 2006; Ferreiro et al., 2008a; Jamieson et al., 2001). However some studies have concluded that cell death is independent of caspase 3 activation (Engelstein et al., 2005; Saez-Valero et al., 2000), as inhibition of caspase 3 was unable to prevent cell death. In contrast we have found that inhibition of caspase 3 results in cell survival in our model (Uppington and Brown, 2008) suggesting that cell death in our model is initiated by caspase 3.

Investigations have revealed several signalling mechanisms by which cell death may occur in prion disease. It has been suggested that cell death is due to endoplasmic reticulum (ER) stress which causes activation of caspase 12, an ER associated caspase (Hetz et al., 2003b). Further work has highlighted the role of calcium dysregulation in apoptosis; cells treated with PrP 106-126 release calcium through ER ryanodine (RyR) and inositol 1,4,5-trisphosphate (IP(3)R) receptors inducing ER stress and leading to increased cytosolic calcium and reactive oxygen species (ROS) levels leading to apoptotic death involving the mitochondria (Ferreiro et al., 2008a; Ferreiro et al., 2008b). However *in vivo* investigations has not shown evidence of ER stress in the brain's of mice infected with scrapie (Unterberger et al., 2006).

Other studies have investigated the role of Bax in prion disease. PrP^c prevents Bax mediated cell death, by preventing the conformational changes that occur upon activation of Bax (Roucou et al., 2005). However Bax has also been implicated in

the neurotoxicity of mutant prion proteins (Chiesa et al., 2005; Li et al., 2007a), but deletion of Bax does not prevent against prion toxicity in mice infected with scrapie (Steele et al., 2007) suggesting that this may not be an obligatory pathway for neuronal apoptosis in prion diseases.

Many studies have investigated the role of the MAPKs and SAPKs in neuronal apoptosis in prion diseases. Studies have suggested that JNK is activated in response to PrP 106-126 (Carimalo et al., 2005; Pietri et al., 2006) and activation may be due to increased oxidative stress (Pietri et al., 2006). JNK activation has also been observed *in vivo* (Lee et al., 2005) and inhibition of JNK activation significantly reduced cell death *in vitro* (Carimalo et al., 2005). p38, a member of the MAPK family, has also been implicated neuronal apoptosis in prion disease. p38 is activated in cells treated with PrP 106-126 and inhibition of p38 prevents PrP 106-126 toxicity (Corsaro et al., 2003). Similar results were also observed in cells treated with the toxic conformer of PrP 90-231 (Villa et al., 2006).

In common with many other studies investigating prion toxicity we found that caspase 3 was activated in response to MnPrP leading to DNA fragmentation (figure 7.1). Further work has revealed that, unlike some studies, caspase 3 inhibition results cell survival, suggesting that caspase 3 activation is required for cell death in response to MnPrP. Investigation has revealed that p38 is also activated in response to MnPrP and we propose that p38 is responsible for the activation of caspase 3. However further work is required to confirm this. Our work is in line with *in vivo* models of prion disease in that cell death is due to apoptosis and this is mediated caspase 3. However as with other models of prion diseases the upstream signalling events leading to caspase 3 activation remains unclear. Further investigation is required to fully understand the upstream signalling that leads to cell death in our model. Cell death signalling may be initiated in response to increase ROS we have observed in cells treated with MnPrP and this should be investigated further.

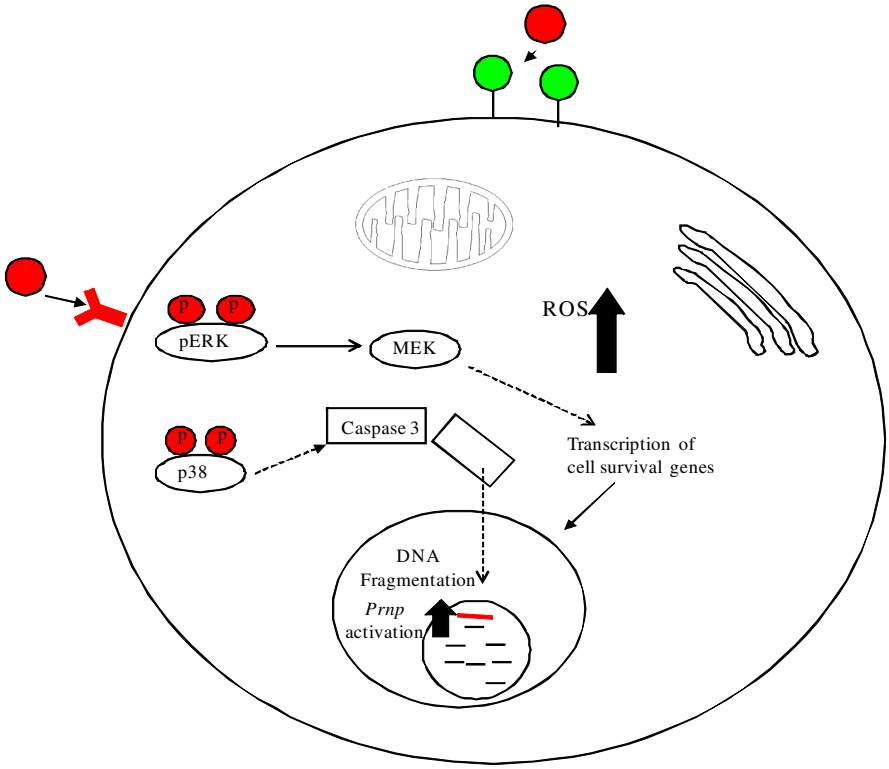


Figure 7.1: A diagram showing the major signalling pathways induced by MnPrP. The red circles represent MnPrP and the green circles represent PrP^{c}, dashed arrows represent assumed pathways.

7.3 Chronic scrapie infection and cell survival

One of the hallmarks of prion disease is vacuolation in the brain caused by massive neuronal cell death (Fairbairn et al., 1994; Giese et al., 1995; Gray et al., 1999), however several cell lines have been shown to be able to maintain scrapie infection with no detrimental effect. Perhaps the best studied is the N2a cell line, which can be infected with scrapie and mouse adapted CJD without any detrimental effect (Butler et al., 1988). SMB cells can also be infected with scrapie and again show no detrimental effects (Birkett et al., 2001). However many cell lines cannot be infected with scrapie, and other cell lines, such as GT1 cells, undergo low-level apoptosis (Schatzl et al., 1997). Interestingly over-expression of Trk4, a receptor for NGF, in GT1 cells and treatment with NGF resulted in a reversal of apoptotic signs (Schatzl et al., 1997). NGF, a cell survival molecule, can signal through ERK and it may be that the up-regulation observed by us in scrapie infected SMB cells, which we propose is a survival mechanism, is similar to the cell survival mechanism observed

in GT1 cells over-expressing Trk 4. There are examples of ERK playing a role in survival in other neurodegenerative diseases. Brain-derived neurotrophic factor (BDNF) has been shown to protect neonatal brains from hypoxic-ischemic injury via an ERK mediated pathway (Han and Holtzman, 2000). Zwitter mice have been suggested to develop neurodegeneration by a decrease in active ERK through intracellular signalling via oxidative stress (Muto and Sato, 2003). D-fructose-1,6-bisphosphate against has been shown to protect against β-amyloid neurotoxicity in rat hippocampal organotypic slice cultures, partially due to the up-regulation of ERK (Song et al., 2005). Further work in an *in vivo* model of prion disease should investigate whether over-expression of ERK can result in an extension of scrapie incubation time.

7.4 Possible therapies for prion diseases

A variety of treatments have been tested both *in vivo* and *in vitro* however, as yet, no treatment for prion disease has been used successfully in humans. Compounds that interact with PrP^{sc} could be useful in preventing conversion by stopping PrP^{sc} interacting with PrP^{c}. Several compounds have been shown to bind PrP and inhibit conversion of PrP. These include Congo red and its derivatives. Congo red is dye used to stain amyloid protein fibrils in histopathology. It has also been shown to delay the onset of prion disease when injected into animals at the time of infection (Ingrosso et al., 1995). However Congo red has several shortfalls including a low permeability to the BBB. Several derivatives of Congo red have been shown to be effective in curing cells *in vitro* and may be more suitable as a therapy (Sellarajah et al., 2004).

Several other compounds have been shown to prevent the conversion of PrP^{c} to PrP^{sc}, quinacrine, an anti-malarial, binds to PrP^{c} and, *in vitro*, was able to inhibit the accumulation of PrP^{sc} in scrapie infected cells (Doh-Ura et al., 2000). However in tests *in vivo* has proved to be ineffective either as a prophylactic (Collins et al., 2002) or in mice already infected with prion disease (Doh-ura et al., 2004). Several patients have also been treated with quinacrine however benefits have been limited (Benito-Leon, 2004; Nakajima et al., 2004). Pentosan polysulphate (PPS) has also been shown to prevent the propagation of PrP^{sc} *in vitro* (Caughey and Raymond, 1993)

and prolong the incubation period of infection *in vivo* (Doh-ura et al., 2004; Ladogana et al., 1992).

An emerging therapy may be RNA aptamers, RNA aptamers are structural pieces of RNA that can interfere with specific proteins. Several aptamers have been shown to specifically bind PrP. And one aptamer, which recognises a peptide comprising amino acid residues 90-129 of the human prion protein with high specificity, has been shown to significantly reduce the *de novo* production of PrP^{sc} *in vitro* (Proske et al., 2002).

Down-regulating cellular prion expression has been proposed as a possible therapy for prion diseases as prion expression is required for infection (Bueler et al., 1993) and down-regulating PrP expression has been shown to lengthen disease incubation time (Manson et al., 1994; Prusiner et al., 1993). Research in mouse models has also shown that down-regulating prion protein expression in mice infected with prion disease halts neuronal cells death and the mouse recovers, showing that even in infected animals, down regulation of PrP expression could lead to a halt in disease progression (Mallucci et al., 2007). We investigated whether MnPrP was able to alter PrP promoter activity, as PrP expression is up-regulated in disease. We found that MnPrP was able to up-regulate promoter activity (although this was not statistically significant) and apoPrP down-regulated promoter activity. This suggests that PrP can regulate its own promoter and further investigation into the mechanisms by which PrP can do this may lead to possible therapeutic target.

In the disease state immunohistochemistry of brain slices of mice infected with scrapie have revealed several markers of oxidative stress including nitrotyrosine, a marker of peroxynitrite generation (Guentchev et al., 2000) and lipid oxidation markers (Wong et al., 2001a). In *vitro* experiments are in agreement with these results, oxidative stress was induced in cells treated with the 106-126 fragment of PrP (Brown et al., 1997b). It is clear that oxidative stress may have a significant role in neurodegeneration in prion disease and prevention of oxidative stress may slow disease progression. However there has been little research into specific antioxidant therapies in CJD, although a single case of treatment with antioxidants has been reported, with an increase in life expectancy (Drisko, 2002). Oxidative stress is also

prominent in both Parkinson's and Alzheimer's, markers of oxidative stress are also found in the brains of Parkinson's sufferers (Jenner et al., 1992) and Alzheimer's sufferers (Sayre et al., 1997). AB and alpha-synuclein have been shown to generate hydrogen peroxide during the early stages of aggregation (Tabner et al., 2005), as has as a fragment of PrP (Turnbull et al., 2003b). It has been proposed that this peroxide generation may mediate toxicity as an antioxidant can reduce the toxicity of PrP 106-126 probably by scavenging hydroxyl radicals produced by it (Turnbull et al., 2003c). Recent work has suggested that attenuation of oxidative stress and mitochondrial dysregulation had therapeutic benefit in an animal model of Parkinson's (Stack et al., 2008). We observed an increase in cellular ROS in cells treated with MnPrP, but treatment with antioxidants did not reduce cell death, however further work should investigate other antioxidants. The mechanisms underlying ROS production by cells treated with MnPrP should also be investigated, this may allow inhibition of ROS production and therefore prevent cell death.

There has been extensive work investigating the species barrier that generally prevents the spread of prion disease between different species. Studies have demonstrated that inoculation of mice with hamster scrapie generally does not result in disease, unless the mice express hamster PrP (Prusiner et al., 1990; Scott et al., 1989). This suggests that expression of PrP may have an inhibitory effect on the conversion of PrP from other species. We investigated whether expressing non-mammalian prion proteins we could prevent the toxicity of MnPrP. We choose to use non-mammalian prion proteins as they show little sequence homology to mouse PrP, although they do retain a surprisingly similar secondary structure (Ahn and Son, 2007; Calzolai et al., 2005). Xenopus PrP does not have the octarepeat region conserved in most prion proteins (Calzolai et al., 2005) and unpublished data from our laboratory has shown that it does not bind metals. Turtle PrP is closer in primary structure to mouse PrP, still retaining some of the metal binding motifs (Calzolai et al., 2005). Neither turtle PrP nor Xenopus PrP expression was able to completely save cells from MnPrP induced cell death, although turtle PrP expression did give a slight protective effect which should be investigated further. Previous work using recombinant protein has demonstrated that a human PrP^{res} seed is unable to convert recombinant mouse PrP to PrP^{res} *in vitro* suggesting that the species barrier still exists between recombinant proteins (Baskakov, 2004). This led us to the conclusion

that the non-mammalian proteins expressed by the cells must still have at least some of the regions required for toxicity, this is perhaps unsurprising given that certain regions have been shown to retain structural motifs that may be important in function. Further work should investigate the regions required for PrP toxicity, possibly by expressing mutants missing regions of the prion protein in PrP-Null cells and treating with MnPrP. Further investigation of the protective effect of turtle PrP should also be undertaken.

This study has demonstrated that manganese bound PrP is a sound model of prion toxicity. It also provided some insight into the mechanisms that lead to MnPrP cell death. However further work is required to produce an animal model of MnPrP infection. Further work should also be carried out in the *in vitro* model to better understand the mechanism of toxicity, this may provide possible therapeutic targets.

References

(1996) From the Centers for Disease Control and Prevention. Surveillance for Creutzfeldt-Jakob disease--United States. *Jama*, **276**, 938-939.

Abdelraheim, S.R., Kralovicova, S. and Brown, D.R. (2006) Hydrogen peroxide cleavage of the prion protein generates a fragment able to initiate polymerisation of full length prion protein. *Int J Biochem Cell Biol*, **38**, 1429-1440.

Aguzzi, A., Raeber, A., Blattler, T., Flechsig, E., Klein, M., Weissmann, C. and Brandner, S. (1997) Neurotoxicity and neuroinvasiveness of prions. *J Neurovirology*, **3 Suppl 1**, S23-24.

Ahn, I. and Son, H.S. (2007) Comparative bioinformatics analysis of prion proteins isolated from reptile, rodent, ruminant, and human species. *Exp Mol Med*, **39**, 769-777.

Ala, A., Walker, A.P., Ashkan, K., Dooley, J.S. and Schilsky, M.L. (2007) Wilson's disease. *Lancet*, **369**, 397-408.

Aronoff-Spencer, E., Burns, C.S., Avdievich, N.I., Gerfen, G.J., Peisach, J., Antholine, W.E., Ball, H.L., Cohen, F.E., Prusiner, S.B. and Millhauser, G.L. (2000) Identification of the Cu2+ binding sites in the N-terminal domain of the prion protein by EPR and CD spectroscopy. *Biochemistry*, **39**, 13760-13771.

Baskakov, I.V. (2004) Autocatalytic conversion of recombinant prion proteins displays a species barrier. *J Biol Chem*, **279**, 7671-7677.

Baskakov, I.V., Legname, G., Baldwin, M.A., Prusiner, S.B. and Cohen, F.E. (2002) Pathway complexity of prion protein assembly into amyloid. *J Biol Chem*, **277**, 21140-21148.

Basu, S., Mohan, M.L., Luo, X., Kundu, B., Kong, Q. and Singh, N. (2007) Modulation of proteinase K-resistant prion protein in cells and infectious brain homogenate by redox iron: implications for prion replication and disease pathogenesis. *Mol Biol Cell*, **18**, 3302-3312.

Baumann, F., Tolnay, M., Brabeck, C., Pahnke, J., Kloz, U., Niemann, H.H., Heikenwalder, M., Rulicke, T., Burkle, A. and Aguzzi, A. (2007) Lethal recessive myelin toxicity of prion protein lacking its central domain. *Embo J*, **26**, 538-547.

Benito-Leon, J. (2004) Combined quinacrine and chlorpromazine therapy in fatal familial insomnia. *Clin Neuropharmacol*, **27**, 201-203.

Biasini, E., Medrano, A.Z., Thellung, S., Chiesa, R. and Harris, D.A. (2008) Multiple biochemical similarities between infectious and non-infectious aggregates of a prion protein carrying an octapeptide insertion. *J Neurochem*, **104**, 1293-1308.

Binolfi, A., Rasia, R.M., Bertoncini, C.W., Ceolin, M., Zweckstetter, M., Griesinger, C., Jovin, T.M. and Fernandez, C.O. (2006) Interaction of alpha-synuclein with divalent metal ions reveals key differences: a link between structure, binding specificity and fibrillation enhancement. *J Am Chem Soc*, **128**, 9893-9901.

Birkett, C.R., Hennion, R.M., Bembridge, D.A., Clarke, M.C., Chree, A., Bruce, M.E. and Bostock, C.J. (2001) Scrapie strains maintain biological phenotypes on propagation in a cell line in culture. *Embo J*, **20**, 3351-3358.

Bocharova, O.V., Breydo, L., Salnikov, V.V. and Baskakov, I.V. (2005) Copper(II) inhibits in vitro conversion of prion protein into amyloid fibrils. *Biochemistry*, **44**, 6776-6787.

Bonetto, V., Massignan, T., Chiesa, R., Morbin, M., Mazzoleni, G., Diomede, L., Angeretti, N., Colombo, L., Forloni, G., Tagliavini, F. and Salmona, M. (2002) Synthetic miniprion PrP106. *J Biol Chem*, **277**, 31327-31334.

Boothby, L.A. and Doering, P.L. (2005) Vitamin C and vitamin E for Alzheimer's disease. *Ann Pharmacother*, **39**, 2073-2080.

Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C. and Aguzzi, A. (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*, **379**, 339-343.

Brazier, M.W., Davies, P., Player, E., Marken, F., Viles, J.H. and Brown, D.R. (2008) Manganese binding to the prion protein. *J Biol Chem*.

Brown, D.R. (1999) Prion protein expression aids cellular uptake and veratridine-induced release of copper. *J Neurosci Res*, **58**, 717-725.

Brown, D.R. (2000a) Altered toxicity of the prion protein peptide PrP106-126 carrying the Ala(117)-->Val mutation. *Biochem J*, **346 Pt 3**, 785-791.

Brown, D.R. (2000b) PrPSc-like prion protein peptide inhibits the function of cellular prion protein. *Biochem J*, **352 Pt 2**, 511-518.

Brown, D.R. (2001) Copper and prion disease. *Brain Res Bull*, **55**, 165-173.

Brown, D.R., Guantieri, V., Grasso, G., Impellizzeri, G., Pappalardo, G. and Rizzarelli, E. (2004) Copper(II) complexes of peptide fragments of the prion protein. Conformation changes induced by copper(II) and the binding motif in C-terminal protein region. *J Inorg Biochem*, **98**, 133-143.

Brown, D.R., Hafiz, F., Glasssmith, L.L., Wong, B.S., Jones, I.M., Clive, C. and Haswell, S.J. (2000) Consequences of manganese replacement of copper for prion protein function and proteinase resistance. *Embo J*, **19**, 1180-1186.

Brown, D.R., Herms, J. and Kretzschmar, H.A. (1994) Mouse cortical cells lacking cellular PrP survive in culture with a neurotoxic PrP fragment. *Neuroreport*, **5**, 2057-2060.

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D. and Kretzschmar, H. (1997a) The cellular prion protein binds copper in vivo. *Nature*, **390**, 684-687.

Brown, D.R., Schmidt, B. and Kretzschmar, H.A. (1996) Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. *Nature*, **380**, 345-347.

Brown, D.R., Schmidt, B. and Kretzschmar, H.A. (1997b) Effects of oxidative stress on prion protein expression in PC12 cells. *Int J Dev Neurosci*, **15**, 961-972.

Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B. and Kretzschmar, H.A. (1997c) Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol*, **146**, 104-112.

Brown, D.R., Wong, B.S., Hafiz, F., Clive, C., Haswell, S.J. and Jones, I.M. (1999) Normal prion protein has an activity like that of superoxide dismutase. *Biochem J*, **344 Pt 1**, 1-5.

Brown, L.R. and Harris, D.A. (2003) Copper and zinc cause delivery of the prion protein from the plasma membrane to a subset of early endosomes and the Golgi. *J Neurochem*, **87**, 353-363.

Brown, P. and Bradley, R. (1998) 1755 and all that: a historical primer of transmissible spongiform encephalopathy. *Bmj*, **317**, 1688-1692.

Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G. and Fraser, H. (1994) Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos Trans R Soc Lond B Biol Sci*, **343**, 405-411.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H. and Bostock, C.J. (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498-501.

Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M. and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*, **416**, 507-511.

Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. *Cell*, **73**, 1339-1347.

Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M. and Weissmann, C. (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature*, **356**, 577-582.

Bueler, H., Raeber, A., Sailer, A., Fischer, M., Aguzzi, A. and Weissmann, C. (1994) High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. *Mol Med*, **1**, 19-30.

Burns, C.S., Aronoff-Spencer, E., Legname, G., Prusiner, S.B., Antholine, W.E., Gerfen, G.J., Peisach, J. and Millhauser, G.L. (2003) Copper coordination in the full-length, recombinant prion protein. *Biochemistry*, **42**, 6794-6803.

Butler, D.A., Scott, M.R., Bockman, J.M., Borchelt, D.R., Taraboulos, A., Hsiao, K.K., Kingsbury, D.T. and Prusiner, S.B. (1988) Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. *J Virol*, **62**, 1558-1564.

Cabral, A.L., Lee, K.S. and Martins, V.R. (2002) Regulation of the cellular prion protein gene expression depends on chromatin conformation. *J Biol Chem*, **277**, 5675-5682.

Calne, D.B., Chu, N.S., Huang, C.C., Lu, C.S. and Olanow, W. (1994) Manganism and idiopathic parkinsonism: similarities and differences. *Neurology*, **44**, 1583-1586.

Calzolai, L., Lysek, D.A., Perez, D.R., Guntert, P. and Wuthrich, K. (2005) Prion protein NMR structures of chickens, turtles, and frogs. *Proc Natl Acad Sci U S A*, **102**, 651-655.

Cardoso, S.M., Rego, A.C., Pereira, C. and Oliveira, C.R. (2005) Protective effect of zinc on amyloid-beta 25-35 and 1-40 mediated toxicity. *Neurotox Res*, **7**, 273-281.

Carimalo, J., Cronier, S., Petit, G., Peyrin, J.M., Boukhtouche, F., Arbez, N., Lemaigre-Dubreuil, Y., Brugg, B. and Miquel, M.C. (2005) Activation of the JNK-c-Jun pathway during the early phase of neuronal apoptosis induced by PrP106-126 and prion infection. *Eur J Neurosci*, **21**, 2311-2319.

Castilla, J., Saa, P., Hetz, C. and Soto, C. (2005) In vitro generation of infectious scrapie prions. *Cell*, **121**, 195-206.

Caughey, B. and Raymond, G.J. (1993) Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J Virol*, **67**, 643-650.

Cereghetti, G.M., Schweiger, A., Glockshuber, R. and Van Doorslaer, S. (2001) Electron paramagnetic resonance evidence for binding of Cu(2+) to the C-terminal domain of the murine prion protein. *Biophys J*, **81**, 516-525.

Cervenakova, L., Buetefisch, C., Lee, H.S., Taller, I., Stone, G., Gibbs, C.J., Jr., Brown, P., Hallett, M. and Goldfarb, L.G. (1999) Novel PRNP sequence variant associated with familial encephalopathy. *Am J Med Genet*, **88**, 653-656.

Chabry, J., Ratsimanohatra, C., Sponne, I., Elena, P.P., Vincent, J.P. and Pillot, T. (2003) In vivo and in vitro neurotoxicity of the human prion protein (PrP) fragment P118-135 independently of PrP expression. *J Neurosci*, **23**, 462-469.

Chattopadhyay, M., Walter, E.D., Newell, D.J., Jackson, P.J., Aronoff-Spencer, E., Peisach, J., Gerfen, G.J., Bennett, B., Antholine, W.E. and Millhauser, G.L. (2005) The octarepeat domain of the prion protein binds Cu(II) with three distinct coordination modes at pH 7.4. *J Am Chem Soc*, **127**, 12647-12656.

Chen, M., Margittai, M., Chen, J. and Langen, R. (2007) Investigation of alpha-synuclein fibril structure by site-directed spin labeling. *J Biol Chem*, **282**, 24970-24979.

Chen, S.G., Teplow, D.B., Parchi, P., Teller, J.K., Gambetti, P. and Autilio-Gambetti, L. (1995) Truncated forms of the human prion protein in normal brain and in prion diseases. *J Biol Chem*, **270**, 19173-19180.

Chiarini, L.B., Freitas, A.R., Zanata, S.M., Brentani, R.R., Martins, V.R. and Linden, R. (2002) Cellular prion protein transduces neuroprotective signals. *Embo J*, **21**, 3317-3326.

Chiesa, R., Piccardo, P., Dossena, S., Nowoslawski, L., Roth, K.A., Ghetti, B. and Harris, D.A. (2005) Bax deletion prevents neuronal loss but not neurological symptoms in a transgenic model of inherited prion disease. *Proc Natl Acad Sci U S A*, **102**, 238-243.

Chiesa, R., Piccardo, P., Ghetti, B. and Harris, D.A. (1998) Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. *Neuron*, **21**, 1339-1351.

Chihota, C.M., Gravenor, M.B. and Baylis, M. (2004) Investigation of trace elements in soil as risk factors in the epidemiology of scrapie. *Vet Rec*, **154**, 809-813.

Chiovitti, K., Corsaro, A., Thellung, S., Villa, V., Paludi, D., D'Arrigo, C., Russo, C., Perico, A., Ianieri, A., Di Cola, D., Vergara, A., Aceto, A. and Florio, T. (2007) Intracellular accumulation of a mild-denatured monomer of the human PrP fragment 90-231, as possible mechanism of its neurotoxic effects. *J Neurochem*, **103**, 2597-2609.

Choi, C.J., Kanthasamy, A., Anantharam, V. and Kanthasamy, A.G. (2006) Interaction of metals with prion protein: possible role of divalent cations in the pathogenesis of prion diseases. *Neurotoxicology*, **27**, 777-787.

Clarke, M.C. and Haig, D.A. (1970) Multiplication of scrapie agent in cell culture. *Res Vet Sci*, **11**, 500-501.

Cobb, N.J., Sonnichsen, F.D., McHaourab, H. and Surewicz, W.K. (2007) Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure. *Proc Natl Acad Sci U S A*, **104**, 18946-18951.

Cohen, F.E., Pan, K.M., Huang, Z., Baldwin, M., Fletterick, R.J. and Prusiner, S.B. (1994) Structural clues to prion replication. *Science*, **264**, 530-531.

Cohen, F.E. and Prusiner, S.B. (1998) Pathologic conformations of prion proteins. *Annu Rev Biochem*, **67**, 793-819.

Collinge, J. (1997) Human prion diseases and bovine spongiform encephalopathy (BSE). *Hum Mol Genet*, **6**, 1699-1705.

Collinge, J., Palmer, M.S., Sidle, K.C., Gowland, I., Medori, R., Ironside, J. and Lantos, P. (1995a) Transmission of fatal familial insomnia to laboratory animals. *Lancet*, **346**, 569-570.

Collinge, J., Palmer, M.S., Sidle, K.C., Hill, A.F., Gowland, I., Meads, J., Asante, E., Bradley, R., Doey, L.J. and Lantos, P.L. (1995b) Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature*, **378**, 779-783.

Collins, S., McLean, C.A. and Masters, C.L. (2001) Gerstmann-Straussler-Scheinker syndrome,fatal familial insomnia, and kuru: a review of these less common human transmissible spongiform encephalopathies. *J Clin Neurosci*, **8**, 387-397.

Collins, S.J., Lewis, V., Brazier, M., Hill, A.F., Fletcher, A. and Masters, C.L. (2002) Quinacrine does not prolong survival in a murine Creutzfeldt-Jakob disease model. *Ann Neurol*, **52**, 503-506.

Combs, C.K., Johnson, D.E., Cannady, S.B., Lehman, T.M. and Landreth, G.E. (1999) Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J Neurosci*, **19**, 928-939.

Cordeiro, Y., Kraineva, J., Gomes, M.P., Lopes, M.H., Martins, V.R., Lima, L.M., Foguel, D., Winter, R. and Silva, J.L. (2005) The amino-terminal PrP domain is crucial to modulate prion misfolding and aggregation. *Biophys J*, **89**, 2667-2676.

Corsaro, A., Paludi, D., Villa, V., D'Arrigo, C., Chiovitti, K., Thellung, S., Russo, C., Di Cola, D., Ballerini, P., Patrone, E., Schettini, G., Aceto, A. and Florio, T. (2006) Conformation dependent pro-apoptotic activity of the recombinant human prion protein fragment 90-231. *Int J Immunopathol Pharmacol*, **19**, 339-356.

Corsaro, A., Thellung, S., Villa, V., Principe, D.R., Paludi, D., Arena, S., Millo, E., Schettini, D., Damonte, G., Aceto, A., Schettini, G. and Florio, T. (2003) Prion protein fragment 106-126 induces a p38 MAP kinase-dependent apoptosis in SH-SY5Y neuroblastoma cells independently from the amyloid fibril formation. *Ann N Y Acad Sci*, **1010**, 610-622.

Cortijo-Arellano, M., Ponce, J., Durany, N. and Cladera, J. (2008) Amyloidogenic properties of the prion protein fragment PrP(185-208): comparison with Alzheimer's peptide Abeta(1-28), influence of heparin and cell toxicity. *Biochem Biophys Res Commun*, **368**, 238-242.

Cregan, S.P., MacLaurin, J.G., Craig, C.G., Robertson, G.S., Nicholson, D.W., Park, D.S. and Slack, R.S. (1999) Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. *J Neurosci*, **19**, 7860-7869.

Cui, T., Daniels, M., Wong, B.S., Li, R., Sy, M.S., Sassoon, J. and Brown, D.R. (2003) Mapping the functional domain of the prion protein. *Eur J Biochem*, **270**, 3368-3376.

Daniels, M., Cereghetti, G.M. and Brown, D.R. (2001) Toxicity of novel C-terminal prion protein fragments and peptides harbouring disease-related C-terminal mutations. *Eur J Biochem*, **268**, 6155-6164.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B. and Greenberg, M.E. (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell*, **6**, 41-51.

DeArmond, S.J., McKinley, M.P., Barry, R.A., Braunfeld, M.B., McColloch, J.R. and Prusiner, S.B. (1985) Identification of prion amyloid filaments in scrapie-infected brain. *Cell*, **41**, 221-235.

DeMarco, M.L. and Daggett, V. (2004) From conversion to aggregation: protofibril formation of the prion protein. *Proc Natl Acad Sci U S A*, **101**, 2293-2298.

Demuro, A., Mina, E., Kayed, R., Milton, S.C., Parker, I. and Glabe, C.G. (2005) Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. *J Biol Chem*, **280**, 17294-17300.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B. and et al. (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol Cell Biol*, **10**, 5772-5781.

Doh-ura, K., Ishikawa, K., Murakami-Kubo, I., Sasaki, K., Mohri, S., Race, R. and Iwaki, T. (2004) Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. *J Virol*, **78**, 4999-5006.

Doh-Ura, K., Iwaki, T. and Caughey, B. (2000) Lysosomotropic agents and cysteine protease inhibitors inhibit scrapie-associated prion protein accumulation. *J Virol*, **74**, 4894-4897.

Donne, D.G., Viles, J.H., Groth, D., Mehlhorn, I., James, T.L., Cohen, F.E., Prusiner, S.B., Wright, P.E. and Dyson, H.J. (1997) Structure of the recombinant full-length hamster prion protein PrP(29-231): the N terminus is highly flexible. *Proc Natl Acad Sci U S A*, **94**, 13452-13457.

Drisko, J.A. (2002) The use of antioxidants in transmissible spongiform encephalopathies: a case report. *J Am Coll Nutr*, **21**, 22-25.

Dupiereux, I., Zorzi, W., Rachidi, W., Zorzi, D., Pierard, O., Lhereux, B., Heinen, E. and Elmoualij, B. (2006) Study on the toxic mechanism of prion protein peptide 106-126 in neuronal and non neuronal cells. *J Neurosci Res*, **84**, 637-646.

Engelstein, R., Grigoriadis, N., Greig, N.H., Ovadia, H. and Gabizon, R. (2005) Inhibition of P53-related apoptosis had no effect on PrP(Sc) accumulation and prion disease incubation time. *Neurobiol Dis*, **18**, 282-285.

Ettaiche, M., Pichot, R., Vincent, J.P. and Chabry, J. (2000) In vivo cytotoxicity of the prion protein fragment 106-126. *J Biol Chem*, **275**, 36487-36490.

Fairbairn, D.W., Carnahan, K.G., Thwaits, R.N., Grigsby, R.V., Holyoak, G.R. and O'Neill, K.L. (1994) Detection of apoptosis induced DNA cleavage in scrapie-infected sheep brain. *FEMS Microbiol Lett*, **115**, 341-346.

Ferreiro, E., Costa, R., Marques, S., Cardoso, S.M., Oliveira, C.R. and Pereira, C.M. (2008a) Involvement of mitochondria in endoplasmic reticulum stress-induced apoptotic cell death pathway triggered by the prion peptide PrP(106-126). *J Neurochem*, **104**, 766-776.

Ferreiro, E., Eufrasio, A., Pereira, C., Oliveira, C.R. and Rego, A.C. (2007) Bcl-2 overexpression protects against amyloid-beta and prion toxicity in GT1-7 neural cells. *J Alzheimers Dis*, **12**, 223-228.

Ferreiro, E., Oliveira, C.R. and Pereira, C.M. (2008b) The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. *Neurobiol Dis*.

Ferreiro, E., Resende, R., Costa, R., Oliveira, C.R. and Pereira, C.M. (2006) An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. *Neurobiol Dis*, **23**, 669-678.

Fioriti, L., Angeretti, N., Colombo, L., De Luigi, A., Colombo, A., Manzoni, C., Morbin, M., Tagliavini, F., Salmona, M., Chiesa, R. and Forloni, G. (2007) Neurotoxic and gliotrophic activity of a synthetic peptide homologous to Gerstmann-Straussler-Scheinker disease amyloid protein. *J Neurosci*, **27**, 1576-1583.

Fioriti, L., Dossena, S., Stewart, L.R., Stewart, R.S., Harris, D.A., Forloni, G. and Chiesa, R. (2005) Cytosolic prion protein (PrP) is not toxic in N2a cells and primary neurons expressing pathogenic PrP mutations. *J Biol Chem*, **280**, 11320-11328.

Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *Embo J*, **15**, 1255-1264.

Flechsig, E., Shmerling, D., Hegyi, I., Raeber, A.J., Fischer, M., Cozzio, A., von Mering, C., Aguzzi, A. and Weissmann, C. (2000) Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron*, **27**, 399-408.

Florent, S., Malaplate-Armand, C., Youssef, I., Kriem, B., Koziel, V., Escanye, M.C., Fifre, A., Sponne, I., Leininger-Muller, B., Olivier, J.L., Pillot, T. and Oster, T. (2006) Docosahexaenoic acid prevents neuronal apoptosis induced by soluble amyloid-beta oligomers. *J Neurochem*, **96**, 385-395.

Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993) Neurotoxicity of a prion protein fragment. *Nature*, **362**, 543-546.

Forloni, G., Bugiani, O., Tagliavini, F. and Salmona, M. (1996) Apoptosis-mediated neurotoxicity induced by beta-amyloid and PrP fragments. *Mol Chem Neuropathol*, **28**, 163-171.

Frankenfield, K.N., Powers, E.T. and Kelly, J.W. (2005) Influence of the N-terminal domain on the aggregation properties of the prion protein. *Protein Sci*, **14**, 2154-2166.

Freixes, M., Rodriguez, A., Dalfo, E. and Ferrer, I. (2006) Oxidation, glycoxidation, lipoxidation, nitration, and responses to oxidative stress in the cerebral cortex in Creutzfeldt-Jakob disease. *Neurobiol Aging*, **27**, 1807-1815.

Gaggelli, E., Bernardi, F., Molteni, E., Pogni, R., Valensin, D., Valensin, G., Remelli, M., Luczkowski, M. and Kozlowski, H. (2005) Interaction of the human prion PrP(106-126) sequence with copper(II), manganese(II), and zinc(II): NMR and EPR studies. *J Am Chem Soc*, **127**, 996-1006.

Garnett, A.P. and Viles, J.H. (2003) Copper binding to the octarepeats of the prion protein. Affinity, specificity, folding, and cooperativity: insights from circular dichroism. *J Biol Chem*, **278**, 6795-6802.

Gasset, M., Baldwin, M.A., Lloyd, D.H., Gabriel, J.M., Holtzman, D.M., Cohen, F., Fletterick, R. and Prusiner, S.B. (1992) Predicted alpha-helical regions of the prion protein when synthesized as peptides form amyloid. *Proc Natl Acad Sci U S A*, **89**, 10940-10944.

Gauczynski, S., Peyrin, J.M., Haik, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J.P., Dormont, D., Lasmezas, C.I. and Weiss, S.

(2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo J*, **20**, 5863-5875.
Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J. and Morrison, R.S. (2000) p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. *J Cell Biol*, **150**, 335-347.
Ghetti, B., Piccardo, P., Frangione, B., Bugiani, O., Giaccone, G., Young, K., Prelli, F., Farlow, M.R., Dlouhy, S.R. and Tagliavini, F. (1996) Prion protein amyloidosis. *Brain Pathol*, **6**, 127-145.
Giese, A., Groschup, M.H., Hess, B. and Kretzschmar, H.A. (1995) Neuronal cell death in scrapie-infected mice is due to apoptosis. *Brain Pathol*, **5**, 213-221.
Giese, A., Levin, J., Bertsch, U. and Kretzschmar, H. (2004) Effect of metal ions on de novo aggregation of full-length prion protein. *Biochem Biophys Res Commun*, **320**, 1240-1246.
Giuffrida, M.L., Grasso, G., Ruvo, M., Pedone, C., Saporito, A., Marasco, D., Pignataro, B., Cascio, C., Copani, A. and Rizzarelli, E. (2007) Abeta(25-35) and its C- and/or N-blocked derivatives: copper driven structural features and neurotoxicity. *J Neurosci Res*, **85**, 623-633.
Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P. and Wolozin, B. (2002) Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. *J Biol Chem*, **277**, 16116-16123.
Govaerts, C., Wille, H., Prusiner, S.B. and Cohen, F.E. (2004) Evidence for assembly of prions with left-handed beta-helices into trimers. *Proc Natl Acad Sci U S A*, **101**, 8342-8347.
Gray, F., Chretien, F., Adle-Biassette, H., Dorandeu, A., Ereau, T., Delisle, M.B., Kopp, N., Ironside, J.W. and Vital, C. (1999) Neuronal apoptosis in Creutzfeldt-Jakob disease. *J Neuropathol Exp Neurol*, **58**, 321-328.
Guentchev, M., Voigtlander, T., Haberler, C., Groschup, M.H. and Budka, H. (2000) Evidence for oxidative stress in experimental prion disease. *Neurobiol Dis*, **7**, 270-273.
Haigh, C.L. and Brown, D.R. (2006) Prion protein reduces both oxidative and non-oxidative copper toxicity. *J Neurochem*, **98**, 677-689.
Haigh, C.L., Edwards, K. and Brown, D.R. (2005) Copper binding is the governing determinant of prion protein turnover. *Mol Cell Neurosci*, **30**, 186-196.
Haigh, C.L., Wright, J.A. and Brown, D.R. (2007) Regulation of prion protein expression by noncoding regions of the Prnp gene. *J Mol Biol*, **368**, 915-927.
Han, B.H. and Holtzman, D.M. (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *J Neurosci*, **20**, 5775-5781.
Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B. and Lingappa, V.R. (1998) A transmembrane form of the prion protein in neurodegenerative disease. *Science*, **279**, 827-834.
Herms, J., Tings, T., Gall, S., Madlung, A., Giese, A., Siebert, H., Schurmann, P., Windl, O., Brose, N. and Kretzschmar, H. (1999) Evidence of presynaptic location and function of the prion protein. *J Neurosci*, **19**, 8866-8875.
Hesketh, S., Sassoon, J., Knight, R. and Brown, D.R. (2008) Elevated manganese levels in blood and CNS in human prion disease. *Mol Cell Neurosci*, **37**, 590-598.
Hesketh, S., Sassoon, J., Knight, R., Hopkins, J. and Brown, D.R. (2007) Elevated manganese levels in blood and central nervous system occur before onset of

clinical signs in scrapie and bovine spongiform encephalopathy. *J Anim Sci*, **85**, 1596-1609.
Hetz, C., Maundrell, K. and Soto, C. (2003a) Is loss of function of the prion protein the cause of prion disorders? *Trends Mol Med*, **9**, 237-243.
Hetz, C., Russelakis-Carneiro, M., Maundrell, K., Castilla, J. and Soto, C. (2003b) Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. *Embo J*, **22**, 5435-5445.
Hetz, C., Russelakis-Carneiro, M., Walchli, S., Carboni, S., Vial-Knecht, E., Maundrell, K., Castilla, J. and Soto, C. (2005) The disulfide isomerase Grp58 is a protective factor against prion neurotoxicity. *J Neurosci*, **25**, 2793-2802.
Hewitt, P.E., Llewelyn, C.A., Mackenzie, J. and Will, R.G. (2006) Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study. *Vox Sang*, **91**, 221-230.
Hijazi, N., Kariv-Inbal, Z., Gasset, M. and Gabizon, R. (2005) PrPSc incorporation to cells requires endogenous glycosaminoglycan expression. *J Biol Chem*, **280**, 17057-17061.
Hijazi, N., Shaked, Y., Rosenmann, H., Ben-Hur, T. and Gabizon, R. (2003) Copper binding to PrPC may inhibit prion disease propagation. *Brain Res*, **993**, 192-200.
Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J. and Lantos, P. (1997) The same prion strain causes vCJD and BSE. *Nature*, **389**, 448-450, 526.
Hill, A.F., Joiner, S., Linehan, J., Desbruslais, M., Lantos, P.L. and Collinge, J. (2000) Species-barrier-independent prion replication in apparently resistant species. *Proc Natl Acad Sci U S A*, **97**, 10248-10253.
Holme, A., Daniels, M., Sassoon, J. and Brown, D.R. (2003) A novel method of generating neuronal cell lines from gene-knockout mice to study prion protein membrane orientation. *Eur J Neurosci*, **18**, 571-579.
Holscher, C., Delius, H. and Burkle, A. (1998) Overexpression of nonconvertible PrPc delta114-121 in scrapie-infected mouse neuroblastoma cells leads to trans-dominant inhibition of wild-type PrP(Sc) accumulation. *J Virol*, **72**, 1153-1159.
Hornemann, S., Korth, C., Oesch, B., Riek, R., Wider, G., Wuthrich, K. and Glockshuber, R. (1997) Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization. *FEBS Lett*, **413**, 277-281.
Hornshaw, M.P., McDermott, J.R. and Candy, J.M. (1995) Copper binding to the N-terminal tandem repeat regions of mammalian and avian prion protein. *Biochem Biophys Res Commun*, **207**, 621-629.
Hsiao, K., Baker, H.F., Crow, T.J., Poulter, M., Owen, F., Terwilliger, J.D., Westaway, D., Ott, J. and Prusiner, S.B. (1989) Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. *Nature*, **338**, 342-345.
Hsiao, K., Scott, M., Foster, D., DeArmond, S.J., Groth, D., Serban, H. and Prusiner, S.B. (1991) Spontaneous neurodegeneration in transgenic mice with prion protein codon 101 proline----leucine substitution. *Ann N Y Acad Sci*, **640**, 166-170.
Hsiao, K.K., Scott, M., Foster, D., Groth, D.F., DeArmond, S.J. and Prusiner, S.B. (1990) Spontaneous neurodegeneration in transgenic mice with mutant prion protein. *Science*, **250**, 1587-1590.

Hsich, G., Kenney, K., Gibbs, C.J., Lee, K.H. and Harrington, M.G. (1996) The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. *N Engl J Med*, **335**, 924-930.

Huang, M.E., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhoa, L., Gu, L.J. and Wang, Z.Y. (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*, **72**, 567-572.

Ingrosso, L., Ladogana, A. and Pocchiari, M. (1995) Congo red prolongs the incubation period in scrapie-infected hamsters. *J Virol*, **69**, 506-508.

Jackson, G.S., Murray, I., Hosszu, L.L., Gibbs, N., Waltho, J.P., Clarke, A.R. and Collinge, J. (2001) Location and properties of metal-binding sites on the human prion protein. *Proc Natl Acad Sci U S A*, **98**, 8531-8535.

Jamieson, E., Jeffrey, M., Ironside, J.W. and Fraser, J.R. (2001) Activation of Fas and caspase 3 precedes PrP accumulation in 87V scrapie. *Neuroreport*, **12**, 3567-3572.

Janicke, R.U., Sprengart, M.L., Wati, M.R. and Porter, A.G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem*, **273**, 9357-9360.

Jenner, P., Dexter, D.T., Sian, J., Schapira, A.H. and Marsden, C.D. (1992) Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. *Ann Neurol*, **32 Suppl**, S82-87.

Jin, T., Gu, Y., Zanusso, G., Sy, M., Kumar, A., Cohen, M., Gambetti, P. and Singh, N. (2000) The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. *J Biol Chem*, **275**, 38699-38704.

Jobling, M.F., Huang, X., Stewart, L.R., Barnham, K.J., Curtain, C., Volitakis, I., Perugini, M., White, A.R., Cherny, R.A., Masters, C.L., Barrow, C.J., Collins, S.J., Bush, A.I. and Cappai, R. (2001) Copper and zinc binding modulates the aggregation and neurotoxic properties of the prion peptide PrP106-126. *Biochemistry*, **40**, 8073-8084.

Jobling, M.F., Stewart, L.R., White, A.R., McLean, C., Friedhuber, A., Maher, F., Beyreuther, K., Masters, C.L., Barrow, C.J., Collins, S.J. and Cappai, R. (1999) The hydrophobic core sequence modulates the neurotoxic and secondary structure properties of the prion peptide 106-126. *J Neurochem*, **73**, 1557-1565.

Jones, C.E., Abdelraheim, S.R., Brown, D.R. and Viles, J.H. (2004) Preferential Cu2+ coordination by His96 and His111 induces beta-sheet formation in the unstructured amyloidogenic region of the prion protein. *J Biol Chem*, **279**, 32018-32027.

Jones, C.E., Klewpatinond, M., Abdelraheim, S.R., Brown, D.R. and Viles, J.H. (2005a) Probing copper2+ binding to the prion protein using diamagnetic nickel2+ and 1H NMR: the unstructured N terminus facilitates the coordination of six copper2+ ions at physiological concentrations. *J Mol Biol*, **346**, 1393-1407.

Jones, E.M., Surewicz, K. and Surewicz, W.K. (2006) Role of N-terminal familial mutations in prion protein fibrillization and prion amyloid propagation in vitro. *J Biol Chem*, **281**, 8190-8196.

Jones, E.M. and Surewicz, W.K. (2005) Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids. *Cell*, **121**, 63-72.

Jones, S., Batchelor, M., Bhelt, D., Clarke, A.R., Collinge, J. and Jackson, G.S. (2005b) Recombinant prion protein does not possess SOD-1 activity. *Biochem J*, **392**, 309-312.

Kaneko, K., Zulianello, L., Scott, M., Cooper, C.M., Wallace, A.C., James, T.L., Cohen, F.E. and Prusiner, S.B. (1997) Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proc Natl Acad Sci U S A*, **94**, 10069-10074.

Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W. and Glabe, C.G. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science*, **300**, 486-489.

Kazlauskaite, J., Young, A., Gardner, C.E., Macpherson, J.V., Venien-Bryan, C. and Pinheiro, T.J. (2005) An unusual soluble beta-turn-rich conformation of prion is involved in fibril formation and toxic to neuronal cells. *Biochem Biophys Res Commun*, **328**, 292-305.

Kim, N.H., Choi, J.K., Jeong, B.H., Kim, J.I., Kwon, M.S., Carp, R.I. and Kim, Y.S. (2005) Effect of transition metals (Mn, Cu, Fe) and deoxycholic acid (DA) on the conversion of PrPC to PrPres. *Faseb J*, **19**, 783-785.

Kim, N.H., Park, S.J., Jin, J.K., Kwon, M.S., Choi, E.K., Carp, R.I. and Kim, Y.S. (2000) Increased ferric iron content and iron-induced oxidative stress in the brains of scrapie-infected mice. *Brain Res*, **884**, 98-103.

Kitamoto, T. and Tateishi, J. (1994) Human prion diseases with variant prion protein. *Philos Trans R Soc Lond B Biol Sci*, **343**, 391-398.

Klajnert, B., Cortijo-Arellano, M., Bryszewska, M. and Cladera, J. (2006) Influence of heparin and dendrimers on the aggregation of two amyloid peptides related to Alzheimer's and prion diseases. *Biochem Biophys Res Commun*, **339**, 577-582.

Klamt, F., Dal-Pizzol, F., Conte da Frota, M.J., Walz, R., Andrades, M.E., da Silva, E.G., Brentani, R.R., Izquierdo, I. and Fonseca Moreira, J.C. (2001) Imbalance of antioxidant defense in mice lacking cellular prion protein. *Free Radic Biol Med*, **30**, 1137-1144.

Klebe, R.J., Chen, T. and Ruddle, F.H. (1970) Controlled production of proliferating somatic cell hybrids. *J Cell Biol*, **45**, 74-82.

Klewpatinond, M., Davies, P., Bowen, S., Brown, D.R. and Viles, J.H. (2008) Deconvoluting the Cu2+ binding modes of full-length prion protein. *J Biol Chem*, **283**, 1870-1881.

Klewpatinond, M. and Viles, J.H. (2007) Fragment length influences affinity for Cu2+ and Ni2+ binding to His96 or His111 of the prion protein and spectroscopic evidence for a multiple histidine binding only at low pH. *Biochem J*, **404**, 393-402.

Krasemann, S., Zerr, I., Weber, T., Poser, S., Kretzschmar, H., Hunsmann, G. and Bodemer, W. (1995) Prion disease associated with a novel nine octapeptide repeat insertion in the PRNP gene. *Brain Res Mol Brain Res*, **34**, 173-176.

Kundu, B., Maiti, N.R., Jones, E.M., Surewicz, K.A., Vanik, D.L. and Surewicz, W.K. (2003) Nucleation-dependent conformational conversion of the Y145Stop variant of human prion protein: structural clues for prion propagation. *Proc Natl Acad Sci U S A*, **100**, 12069-12074.

Ladogana, A., Casaccia, P., Ingrosso, L., Cibati, M., Salvatore, M., Xi, Y.G., Masullo, C. and Pocchiari, M. (1992) Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *J Gen Virol*, **73 (Pt 3)**, 661-665.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
Laws, D.D., Bitter, H.M., Liu, K., Ball, H.L., Kaneko, K., Wille, H., Cohen, F.E., Prusiner, S.B., Pines, A. and Wemmer, D.E. (2001) Solid-state NMR studies of the secondary structure of a mutant prion protein fragment of 55 residues that induces neurodegeneration. *Proc Natl Acad Sci U S A*, **98**, 11686-11690.
Lawson, V.A., Priola, S.A., Wehrly, K. and Chesebro, B. (2001) N-terminal truncation of prion protein affects both formation and conformation of abnormal protease-resistant prion protein generated in vitro. *J Biol Chem*, **276**, 35265-35271.
Lee, H.P., Jun, Y.C., Choi, J.K., Kim, J.I., Carp, R.I. and Kim, Y.S. (2005) Activation of mitogen-activated protein kinases in hamster brains infected with 263K scrapie agent. *J Neurochem*, **95**, 584-593.
Lee, I.Y., Westaway, D., Smit, A.F., Wang, K., Seto, J., Chen, L., Acharya, C., Ankener, M., Baskin, D., Cooper, C., Yao, H., Prusiner, S.B. and Hood, L.E. (1998) Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. *Genome Res*, **8**, 1022-1037.
Legleiter, L.R., Ahola, J.K., Engle, T.E. and Spears, J.W. (2007a) Decreased brain copper due to copper deficiency has no effect on bovine prion proteins. *Biochem Biophys Res Commun*, **352**, 884-888.
Legleiter, L.R., Liu, H.C., Lloyd, K.E., Hansen, S.L., Fry, R.S. and Spears, J.W. (2007b) Exposure to low dietary copper or low copper coupled with high dietary manganese for one year does not alter brain prion protein characteristics in the mature cow. *J Anim Sci*, **85**, 2895-2903.
Leucht, C., Simoneau, S., Rey, C., Vana, K., Rieger, R., Lasmezas, C.I. and Weiss, S. (2003) The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep*, **4**, 290-295.
Levin, J., Bertsch, U., Kretzschmar, H. and Giese, A. (2005) Single particle analysis of manganese-induced prion protein aggregates. *Biochem Biophys Res Commun*, **329**, 1200-1207.
Li, A., Barmada, S.J., Roth, K.A. and Harris, D.A. (2007a) N-terminally deleted forms of the prion protein activate both Bax-dependent and Bax-independent neurotoxic pathways. *J Neurosci*, **27**, 852-859.
Li, A., Christensen, H.M., Stewart, L.R., Roth, K.A., Chiesa, R. and Harris, D.A. (2007b) Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105-125. *Embo J*, **26**, 548-558.
Li, G. and Bolton, D.C. (1997) A novel hamster prion protein mRNA contains an extra exon: increased expression in scrapie. *Brain Res*, **751**, 265-274.
Lopez Garcia, F., Zahn, R., Riek, R. and Wuthrich, K. (2000) NMR structure of the bovine prion protein. *Proc Natl Acad Sci U S A*, **97**, 8334-8339.
Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L. and Markesbery, W.R. (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci*, **158**, 47-52.
Lu, X., Wintrode, P.L. and Surewicz, W.K. (2007) Beta-sheet core of human prion protein amyloid fibrils as determined by hydrogen/deuterium exchange. *Proc Natl Acad Sci U S A*, **104**, 1510-1515.
Makarava, N., Lee, C.I., Ostapchenko, V.G. and Baskakov, I.V. (2007) Highly promiscuous nature of prion polymerization. *J Biol Chem*, **282**, 36704-36713.
Mallucci, G.R., White, M.D., Farmer, M., Dickinson, A., Khatun, H., Powell, A.D., Brandner, S., Jefferys, J.G. and Collinge, J. (2007) Targeting cellular prion

protein reverses early cognitive deficits and neurophysiological dysfunction in prion-infected mice. *Neuron*, **53**, 325-335.
Manetto, V., Medori, R., Cortelli, P., Montagna, P., Tinuper, P., Baruzzi, A., Rancurel, G., Hauw, J.J., Vanderhaeghen, J.J., Mailleux, P. and et al. (1992) Fatal familial insomnia: clinical and pathologic study of five new cases. *Neurology*, **42**, 312-319.
Mange, A., Beranger, F., Peoc'h, K., Onodera, T., Frobert, Y. and Lehmann, S. (2004) Alpha- and beta- cleavages of the amino-terminus of the cellular prion protein. *Biol Cell*, **96**, 125-132.
Manson, J.C., Clarke, A.R., McBride, P.A., McConnell, I. and Hope, J. (1994) PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. *Neurodegeneration*, **3**, 331-340.
Manuelidis, L., Fritch, W. and Xi, Y.G. (1997) Evolution of a strain of CJD that induces BSE-like plaques. *Science*, **277**, 94-98.
Martin, B.D., Schoenhard, J.A. and Sugden, K.D. (1998) Hypervalent chromium mimics reactive oxygen species as measured by the oxidant-sensitive dyes 2',7'-dichlorofluorescin and dihydrorhodamine. *Chem Res Toxicol*, **11**, 1402-1410.
Martin, L.J., Pan, Y., Price, A.C., Sterling, W., Copeland, N.G., Jenkins, N.A., Price, D.L. and Lee, M.K. (2006) Parkinson's disease alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death. *J Neurosci*, **26**, 41-50.
Martins, S.M., Frosoni, D.J., Martinez, A.M., De Felice, F.G. and Ferreira, S.T. (2006) Formation of soluble oligomers and amyloid fibrils with physical properties of the scrapie isoform of the prion protein from the C-terminal domain of recombinant murine prion protein mPrP-(121-231). *J Biol Chem*, **281**, 26121-26128.
McLennan, N.F., Brennan, P.M., McNeill, A., Davies, I., Fotheringham, A., Rennison, K.A., Ritchie, D., Brannan, F., Head, M.W., Ironside, J.W., Williams, A. and Bell, J.E. (2004) Prion protein accumulation and neuroprotection in hypoxic brain damage. *Am J Pathol*, **165**, 227-235.
McMahon, H.E., Mange, A., Nishida, N., Creminon, C., Casanova, D. and Lehmann, S. (2001) Cleavage of the amino terminus of the prion protein by reactive oxygen species. *J Biol Chem*, **276**, 2286-2291.
Menkes, J.H., Alter, M., Steigleder, G.K., Weakley, D.R. and Sung, J.H. (1962) A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. *Pediatrics*, **29**, 764-779.
Miura, T., Hori-i, A. and Takeuchi, H. (1996) Metal-dependent alpha-helix formation promoted by the glycine-rich octapeptide region of prion protein. *FEBS Lett*, **396**, 248-252.
Moriya, R., Uehara, T. and Nomura, Y. (2000) Mechanism of nitric oxide-induced apoptosis in human neuroblastoma SH-SY5Y cells. *FEBS Lett*, **484**, 253-260.
Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J.L., Lehmann, S., Launay, J.M. and Kellermann, O. (2000) Signal transduction through prion protein. *Science*, **289**, 1925-1928.
Munoz, L., Ranaivo, H.R., Roy, S.M., Hu, W., Craft, J.M., McNamara, L.K., Chico, L.W., Van Eldik, L.J. and Watterson, D.M. (2007) A novel p38 alpha MAPK inhibitor suppresses brain proinflammatory cytokine up-regulation and attenuates synaptic dysfunction and behavioral deficits in an Alzheimer's disease mouse model. *J Neuroinflammation*, **4**, 21.

Muramoto, T., DeArmond, S.J., Scott, M., Telling, G.C., Cohen, F.E. and Prusiner, S.B. (1997) Heritable disorder resembling neuronal storage disease in mice expressing prion protein with deletion of an alpha-helix. *Nat Med*, **3**, 750-755.

Muramoto, T., Scott, M., Cohen, F.E. and Prusiner, S.B. (1996) Recombinant scrapie-like prion protein of 106 amino acids is soluble. *Proc Natl Acad Sci U S A*, **93**, 15457-15462.

Murray, B., Alessandrini, A., Cole, A.J., Yee, A.G. and Furshpan, E.J. (1998) Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. *Proc Natl Acad Sci U S A*, **95**, 11975-11980.

Muto, Y. and Sato, K. (2003) Pivotal role of attractin in cell survival under oxidative stress in the zitter rat brain with genetic spongiform encephalopathy. *Brain Res Mol Brain Res*, **111**, 111-122.

Nadal, R.C., Abdelraheim, S.R., Brazier, M.W., Rigby, S.E., Brown, D.R. and Viles, J.H. (2007) Prion protein does not redox-silence Cu2+, but is a sacrificial quencher of hydroxyl radicals. *Free Radic Biol Med*, **42**, 79-89.

Nakajima, M., Yamada, T., Kusuhara, T., Furukawa, H., Takahashi, M., Yamauchi, A. and Kataoka, Y. (2004) Results of quinacrine administration to patients with Creutzfeldt-Jakob disease. *Dement Geriatr Cogn Disord*, **17**, 158-163.

Nicolas, O., Gavin, R., Braun, N., Urena, J.M., Fontana, X., Soriano, E., Aguzzi, A. and del Rio, J.A. (2007) Bcl-2 overexpression delays caspase-3 activation and rescues cerebellar degeneration in prion-deficient mice that overexpress amino-terminally truncated prion. *Faseb J*, **21**, 3107-3117.

Nishida, N., Harris, D.A., Vilette, D., Laude, H., Frobert, Y., Grassi, J., Casanova, D., Milhavet, O. and Lehmann, S. (2000) Successful transmission of three mouse-adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild-type mouse prion protein. *J Virol*, **74**, 320-325.

Norstrom, E.M., Ciaccio, M.F., Rassbach, B., Wollmann, R. and Mastrianni, J.A. (2007) Cytosolic prion protein toxicity is independent of cellular prion protein expression and prion propagation. *J Virol*, **81**, 2831-2837.

Norstrom, E.M. and Mastrianni, J.A. (2005) The AGAAAAGA palindrome in PrP is required to generate a productive PrPSc-PrPC complex that leads to prion propagation. *J Biol Chem*, **280**, 27236-27243.

Novitskaya, V., Bocharova, O.V., Bronstein, I. and Baskakov, I.V. (2006) Amyloid fibrils of mammalian prion protein are highly toxic to cultured cells and primary neurons. *J Biol Chem*, **281**, 13828-13836.

Novitskaya, V., Makarava, N., Sylvester, I., Bronstein, I.B. and Baskakov, I.V. (2007) Amyloid fibrils of mammalian prion protein induce axonal degeneration in NTERA2-derived terminally differentiated neurons. *J Neurochem*, **102**, 398-407.

Nunziante, M., Gilch, S. and Schatzl, H.M. (2003) Essential role of the prion protein N terminus in subcellular trafficking and half-life of cellular prion protein. *J Biol Chem*, **278**, 3726-3734.

Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M. and Wolozin, B. (2000) The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. *J Neurosci*, **20**, 6048-6054.

O'Sullivan, D.B., Jones, C.E., Abdelraheim, S.R., Thompsett, A.R., Brazier, M.W., Toms, H., Brown, D.R. and Viles, J.H. (2007) NMR characterization of the pH 4 beta-intermediate of the prion protein: the N-terminal half of the protein

remains unstructured and retains a high degree of flexibility. *Biochem J*, **401**, 533-540.
Paitel, E., Sunyach, C., Alves da Costa, C., Bourdon, J.C., Vincent, B. and Checler, F. (2004) Primary cultured neurons devoid of cellular prion display lower responsiveness to staurosporine through the control of p53 at both transcriptional and post-transcriptional levels. *J Biol Chem*, **279**, 612-618.
Palmer, M.S., Dryden, A.J., Hughes, J.T. and Collinge, J. (1991) Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. *Nature*, **352**, 340-342.
Pan, K.M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. and et al. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A*, **90**, 10962-10966.
Pan, T., Wong, B.S., Liu, T., Li, R., Petersen, R.B. and Sy, M.S. (2002) Cell-surface prion protein interacts with glycosaminoglycans. *Biochem J*, **368**, 81-90.
Pankiewicz, J., Prelli, F., Sy, M.S., Kascsak, R.J., Kascsak, R.B., Spinner, D.S., Carp, R.I., Meeker, H.C., Sadowski, M. and Wisniewski, T. (2006) Clearance and prevention of prion infection in cell culture by anti-PrP antibodies. *Eur J Neurosci*, **23**, 2635-2647.
Pauly, P.C. and Harris, D.A. (1998) Copper stimulates endocytosis of the prion protein. *J Biol Chem*, **273**, 33107-33110.
Pietri, M., Caprini, A., Mouillet-Richard, S., Pradines, E., Ermonval, M., Grassi, J., Kellermann, O. and Schneider, B. (2006) Overstimulation of PrPC signaling pathways by prion peptide 106-126 causes oxidative injury of bioaminergic neuronal cells. *J Biol Chem*, **281**, 28470-28479.
Post, K., Brown, D.R., Groschup, M., Kretzschmar, H.A. and Riesner, D. (2000) Neurotoxicity but not infectivity of prion proteins can be induced reversibly in vitro. *Arch Virol Suppl*, 265-273.
Proske, D., Gilch, S., Wopfner, F., Schatzl, H.M., Winnacker, E.L. and Famulok, M. (2002) Prion-protein-specific aptamer reduces PrPSc formation. *Chembiochem*, **3**, 717-725.
Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. *Science*, **216**, 136-144.
Prusiner, S.B., Groth, D., Serban, A., Koehler, R., Foster, D., Torchia, M., Burton, D., Yang, S.L. and DeArmond, S.J. (1993) Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proc Natl Acad Sci U S A*, **90**, 10608-10612.
Prusiner, S.B., McKinley, M.P., Bowman, K.A., Bolton, D.C., Bendheim, P.E., Groth, D.F. and Glenner, G.G. (1983) Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell*, **35**, 349-358.
Prusiner, S.B., Scott, M., Foster, D., Pan, K.M., Groth, D., Mirenda, C., Torchia, M., Yang, S.L., Serban, D., Carlson, G.A. and et al. (1990) Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell*, **63**, 673-686.
Qin, K., Yang, D.S., Yang, Y., Chishti, M.A., Meng, L.J., Kretzschmar, H.A., Yip, C.M., Fraser, P.E. and Westaway, D. (2000) Copper(II)-induced conformational changes and protease resistance in recombinant and cellular PrP. Effect of protein age and deamidation. *J Biol Chem*, **275**, 19121-19131.

Quaglio, E., Chiesa, R. and Harris, D.A. (2001) Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform. *J Biol Chem*, **276**, 11432-11438.

Race, R.E., Fadness, L.H. and Chesebro, B. (1987) Characterization of scrapie infection in mouse neuroblastoma cells. *J Gen Virol*, **68 (Pt 5)**, 1391-1399.

Rachidi, W., Vilette, D., Guiraud, P., Arlotto, M., Riondel, J., Laude, H., Lehmann, S. and Favier, A. (2003) Expression of prion protein increases cellular copper binding and antioxidant enzyme activities but not copper delivery. *J Biol Chem*, **278**, 9064-9072.

Raeber, A.J., Race, R.E., Brandner, S., Priola, S.A., Sailer, A., Bessen, R.A., Mucke, L., Manson, J., Aguzzi, A., Oldstone, M.B., Weissmann, C. and Chesebro, B. (1997) Astrocyte-specific expression of hamster prion protein (PrP) renders PrP knockout mice susceptible to hamster scrapie. *Embo J*, **16**, 6057-6065.

Rao, R.V., Peel, A., Logvinova, A., del Rio, G., Hermel, E., Yokota, T., Goldsmith, P.C., Ellerby, L.M., Ellerby, H.M. and Bredesen, D.E. (2002) Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. *FEBS Lett*, **514**, 122-128.

Rasia, R.M., Bertoncini, C.W., Marsh, D., Hoyer, W., Cherny, D., Zweckstetter, M., Griesinger, C., Jovin, T.M. and Fernandez, C.O. (2005) Structural characterization of copper(II) binding to alpha-synuclein: Insights into the bioinorganic chemistry of Parkinson's disease. *Proc Natl Acad Sci U S A*, **102**, 4294-4299.

Requena, J.R., Groth, D., Legname, G., Stadtman, E.R., Prusiner, S.B. and Levine, R.L. (2001) Copper-catalyzed oxidation of the recombinant SHa(29-231) prion protein. *Proc Natl Acad Sci U S A*, **98**, 7170-7175.

Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R. and Wuthrich, K. (1996) NMR structure of the mouse prion protein domain PrP(121-321). *Nature*, **382**, 180-182.

Rizzardini, M., Chiesa, R., Angeretti, N., Lucca, E., Salmona, M., Forloni, G. and Cantoni, L. (1997) Prion protein fragment 106-126 differentially induces heme oxygenase-1 mRNA in cultured neurons and astroglial cells. *J Neurochem*, **68**, 715-720.

Roth, J.A., Feng, L., Walowitz, J. and Browne, R.W. (2000) Manganese-induced rat pheochromocytoma (PC12) cell death is independent of caspase activation. *J Neurosci Res*, **61**, 162-171.

Roucou, X., Giannopoulos, P.N., Zhang, Y., Jodoin, J., Goodyer, C.G. and LeBlanc, A. (2005) Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells. *Cell Death Differ*, **12**, 783-795.

Rybner, C., Hillion, J., Sahraoui, T., Lanotte, M. and Botti, J. (2002) All-trans retinoic acid down-regulates prion protein expression independently of granulocyte maturation. *Leukemia*, **16**, 940-948.

Saborio, G.P., Permanne, B. and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature*, **411**, 810-813.

Saeki, K., Matsumoto, Y., Hirota, Y. and Onodera, T. (1996a) Three-exon structure of the gene encoding the rat prion protein and its expression in tissues. *Virus Genes*, **12**, 15-20.

Saeki, K., Matsumoto, Y. and Onodera, T. (1996b) Identification of a promoter region in the rat prion protein gene. *Biochem Biophys Res Commun*, **219**, 47-52.

Saez-Valero, J., Angeretti, N. and Forloni, G. (2000) Caspase-3 activation by beta-amyloid and prion protein peptides is independent from their neurotoxic effect. *Neurosci Lett*, **293**, 207-210.

Sakudo, A., Wu, G., Onodera, T. and Ikuta, K. (2008) Octapeptide repeat region of prion protein (PrP) is required at an early stage for production of abnormal prion protein in PrP-deficient neuronal cell line. *Biochem Biophys Res Commun*, **365**, 164-169.

Salmona, M., Morbin, M., Massignan, T., Colombo, L., Mazzoleni, G., Capobianco, R., Diomede, L., Thaler, F., Mollica, L., Musco, G., Kourie, J.J., Bugiani, O., Sharma, D., Inouye, H., Kirschner, D.A., Forloni, G. and Tagliavini, F. (2003) Structural properties of Gerstmann-Straussler-Scheinker disease amyloid protein. *J Biol Chem*, **278**, 48146-48153.

Sanchez-Alavez, M., Conti, B., Moroncini, G. and Criado, J.R. (2007) Contributions of neuronal prion protein on sleep recovery and stress response following sleep deprivation. *Brain Res*, **1158**, 71-80.

Satoh, J., Obayashi, S., Misawa, T., Sumiyoshi, K., Oosumi, K. and Tabunoki, H. (2008) Protein microarray analysis identifies human cellular prion protein interactors. *Neuropathol Appl Neurobiol*.

Sayre, L.M., Zelasko, D.A., Harris, P.L., Perry, G., Salomon, R.G. and Smith, M.A. (1997) 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J Neurochem*, **68**, 2092-2097.

Schatzl, H.M., Laszlo, L., Holtzman, D.M., Tatzelt, J., DeArmond, S.J., Weiner, R.I., Mobley, W.C. and Prusiner, S.B. (1997) A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis. *J Virol*, **71**, 8821-8831.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S.J. and et al. (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell*, **59**, 847-857.

Sellarajah, S., Lekishvili, T., Bowring, C., Thompsett, A.R., Rudyk, H., Birkett, C.R., Brown, D.R. and Gilbert, I.H. (2004) Synthesis of analogues of Congo red and evaluation of their anti-prion activity. *J Med Chem*, **47**, 5515-5534.

Selvaggini, C., De Gioia, L., Cantu, L., Ghibaudi, E., Diomede, L., Passerini, F., Forloni, G., Bugiani, O., Tagliavini, F. and Salmona, M. (1993) Molecular characteristics of a protease-resistant, amyloidogenic and neurotoxic peptide homologous to residues 106-126 of the prion protein. *Biochem Biophys Res Commun*, **194**, 1380-1386.

Shaked, G.M., Meiner, Z., Avraham, I., Taraboulos, A. and Gabizon, R. (2001) Reconstitution of prion infectivity from solubilized protease-resistant PrP and nonprotein components of prion rods. *J Biol Chem*, **276**, 14324-14328.

Shmerling, D., Hegyi, I., Fischer, M., Blattler, T., Brandner, S., Gotz, J., Rulicke, T., Flechsig, E., Cozzio, A., von Mering, C., Hangartner, C., Aguzzi, A. and Weissmann, C. (1998) Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell*, **93**, 203-214.

Shyu, W.C., Harn, H.J., Saeki, K., Kubosaki, A., Matsumoto, Y., Onodera, T., Chen, C.J., Hsu, Y.D. and Chiang, Y.H. (2002) Molecular modulation of expression of prion protein by heat shock. *Mol Neurobiol*, **26**, 1-12.

Shyu, W.C., Kao, M.C., Chou, W.Y., Hsu, Y.D. and Soong, B.W. (2000) Heat shock modulates prion protein expression in human NT-2 cells. *Neuroreport*, **11**, 771-774.

Shyu, W.C., Lin, S.Z., Saeki, K., Kubosaki, A., Matsumoto, Y., Onodera, T., Chiang, M.F., Thajeb, P. and Li, H. (2004) Hyperbaric oxygen enhances the expression of prion protein and heat shock protein 70 in a mouse neuroblastoma cell line. *Cell Mol Neurobiol*, **24**, 257-268.

Sigurdsson, E.M., Brown, D.R., Alim, M.A., Scholtzova, H., Carp, R., Meeker, H.C., Prelli, F., Frangione, B. and Wisniewski, T. (2003) Copper chelation delays the onset of prion disease. *J Biol Chem*, **278**, 46199-46202.

Simoneau, S., Rezaei, H., Sales, N., Kaiser-Schulz, G., Lefebvre-Roque, M., Vidal, C., Fournier, J.G., Comte, J., Wopfner, F., Grosclaude, J., Schatzl, H. and Lasmezas, C.I. (2007) In vitro and in vivo neurotoxicity of prion protein oligomers. *PLoS Pathog*, **3**, e125.

Solforosi, L., Criado, J.R., McGavern, D.B., Wirz, S., Sanchez-Alavez, M., Sugama, S., DeGiorgio, L.A., Volpe, B.T., Wiseman, E., Abalos, G., Masliah, E., Gilden, D., Oldstone, M.B., Conti, B. and Williamson, R.A. (2004) Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science*, **303**, 1514-1516.

Song, X., Wu, B., Takata, T., Wang, X., Oizumi, X.S., Akisaki, T., Yokono, K. and Sakurai, T. (2005) Neuroprotective effect of D-fructose-1,6-bisphosphate against beta-amyloid induced neurotoxicity in rat hippocampal organotypic slice culture: involvement of PLC and MEK/ERK signaling pathways. *Kobe J Med Sci*, **51**, 73-83.

Sorensen, G., Medina, S., Parchaliuk, D., Phillipson, C., Robertson, C. and Booth, S.A. (2008) Comprehensive transcriptional profiling of prion infection in mouse models reveals networks of responsive genes. *BMC Genomics*, **9**, 114.

Stack, E.C., Ferro, J.L., Kim, J., Del Signore, S.J., Goodrich, S., Matson, S., Hunt, B.B., Cormier, K., Smith, K., Matson, W.R., Ryu, H. and Ferrante, R.J. (2008) Therapeutic attenuation of mitochondrial dysfunction and oxidative stress in neurotoxin models of Parkinson's disease. *Biochim Biophys Acta*, **1782**, 151-162.

Stahl, N., Borchelt, D.R., Hsiao, K. and Prusiner, S.B. (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell*, **51**, 229-240.

Steele, A.D., King, O.D., Jackson, W.S., Hetz, C.A., Borkowski, A.W., Thielen, P., Wollmann, R. and Lindquist, S. (2007) Diminishing apoptosis by deletion of Bax or overexpression of Bcl-2 does not protect against infectious prion toxicity in vivo. *J Neurosci*, **27**, 13022-13027.

Stockel, J., Safar, J., Wallace, A.C., Cohen, F.E. and Prusiner, S.B. (1998) Prion protein selectively binds copper(II) ions. *Biochemistry*, **37**, 7185-7193.

Stohr, J., Weinmann, N., Wille, H., Kaimann, T., Nagel-Steger, L., Birkmann, E., Panza, G., Prusiner, S.B., Eigen, M. and Riesner, D. (2008) Mechanisms of prion protein assembly into amyloid. *Proc Natl Acad Sci U S A*, **105**, 2409-2414.

Stuermer, C.A., Langhorst, M.F., Wiechers, M.F., Legler, D.F., Von Hanwehr, S.H., Guse, A.H. and Plattner, H. (2004) PrPc capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. *Faseb J*, **18**, 1731-1733.

Sunyach, C. and Checler, F. (2005) Combined pharmacological, mutational and cell biology approaches indicate that p53-dependent caspase 3 activation

triggered by cellular prion is dependent on its endocytosis. *J Neurochem*, **92**, 1399-1407.

Sunyach, C., Cisse, M.A., da Costa, C.A., Vincent, B. and Checler, F. (2007) The C-terminal products of cellular prion protein processing, C1 and C2, exert distinct influence on p53-dependent staurosporine-induced caspase-3 activation. *J Biol Chem*, **282**, 1956-1963.

Supattapone, S., Bosque, P., Muramoto, T., Wille, H., Aagaard, C., Peretz, D., Nguyen, H.O., Heinrich, C., Torchia, M., Safar, J., Cohen, F.E., DeArmond, S.J., Prusiner, S.B. and Scott, M. (1999) Prion protein of 106 residues creates an artifical transmission barrier for prion replication in transgenic mice. *Cell*, **96**, 869-878.

Tabner, B.J., Turnbull, S., El-Agnaf, O. and Allsop, D. (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem*, **1**, 507-517.

Tabner, B.J., Turnbull, S., Fullwood, N.J., German, M. and Allsop, D. (2005) The production of hydrogen peroxide during early-stage protein aggregation: a common pathological mechanism in different neurodegenerative diseases? *Biochem Soc Trans*, **33**, 548-550.

Tagami, M., Yamagata, K., Ikeda, K., Nara, Y., Fujino, H., Kubota, A., Numano, F. and Yamori, Y. (1998) Vitamin E prevents apoptosis in cortical neurons during hypoxia and oxygen reperfusion. *Lab Invest*, **78**, 1415-1429.

Talmard, C., Bouzan, A. and Faller, P. (2007) Zinc binding to amyloid-beta: isothermal titration calorimetry and Zn competition experiments with Zn sensors. *Biochemistry*, **46**, 13658-13666.

Tamagno, E., Parola, M., Guglielmotto, M., Santoro, G., Bardini, P., Marra, L., Tabaton, M. and Danni, O. (2003a) Multiple signaling events in amyloid beta-induced, oxidative stress-dependent neuronal apoptosis. *Free Radic Biol Med*, **35**, 45-58.

Tamagno, E., Robino, G., Obbili, A., Bardini, P., Aragno, M., Parola, M. and Danni, O. (2003b) H2O2 and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK. *Exp Neurol*, **180**, 144-155.

Telling, G.C., Scott, M., Hsiao, K.K., Foster, D., Yang, S.L., Torchia, M., Sidle, K.C., Collinge, J., DeArmond, S.J. and Prusiner, S.B. (1994) Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc Natl Acad Sci U S A*, **91**, 9936-9940.

Telling, G.C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F.E., DeArmond, S.J. and Prusiner, S.B. (1995) Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell*, **83**, 79-90.

Thackray, A.M., Knight, R., Haswell, S.J., Bujdoso, R. and Brown, D.R. (2002) Metal imbalance and compromised antioxidant function are early changes in prion disease. *Biochem J*, **362**, 253-258.

Thellung, S., Corsaro, A., Villa, V., Venezia, V., Nizzari, M., Bisaglia, M., Russo, C., Schettini, G., Aceto, A. and Florio, T. (2007) Amino-terminally truncated prion protein PrP90-231 induces microglial activation in vitro. *Ann N Y Acad Sci*, **1096**, 258-270.

Thellung, S., Villa, V., Corsaro, A., Arena, S., Millo, E., Damonte, G., Benatti, U., Tagliavini, F., Florio, T. and Schettini, G. (2002) p38 MAP kinase mediates the cell death induced by PrP106-126 in the SH-SY5Y neuroblastoma cells. *Neurobiol Dis*, **9**, 69-81.

Thompsett, A.R., Abdelraheim, S.R., Daniels, M. and Brown, D.R. (2005) High affinity binding between copper and full-length prion protein identified by two different techniques. *J Biol Chem*, **280**, 42750-42758.

Thompson, A., White, A.R., McLean, C., Masters, C.L., Cappai, R. and Barrow, C.J. (2000) Amyloidogenicity and neurotoxicity of peptides corresponding to the helical regions of PrP(C). *J Neurosci Res*, **62**, 293-301.

Tobler, I., Deboer, T. and Fischer, M. (1997) Sleep and sleep regulation in normal and prion protein-deficient mice. *J Neurosci*, **17**, 1869-1879.

Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., Moser, M., Oesch, B., McBride, P.A. and Manson, J.C. (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature*, **380**, 639-642.

Toni, M., Massimino, M.L., Griffoni, C., Salvato, B., Tomasi, V. and Spisni, E. (2005) Extracellular copper ions regulate cellular prion protein (PrPC) expression and metabolism in neuronal cells. *FEBS Lett*, **579**, 741-744.

Torok, M., Milton, S., Kayed, R., Wu, P., McIntire, T., Glabe, C.G. and Langen, R. (2002) Structural and dynamic features of Alzheimer's Abeta peptide in amyloid fibrils studied by site-directed spin labeling. *J Biol Chem*, **277**, 40810-40815.

Tortosa, R., Vidal, E., Costa, C., Alamillo, E., Torres, J.M., Ferrer, I. and Pumarola, M. (2007) Stress response in the central nervous system of a transgenic mouse model of bovine spongiform encephalopathy. *Vet J*.

Tougu, V., Karafin, A. and Palumaa, P. (2008) Binding of zinc(II) and copper(II) to the full-length Alzheimer's amyloid-beta peptide. *J Neurochem*, **104**, 1249-1259.

Treiber, C., Simons, A. and Multhaup, G. (2006) Effect of copper and manganese on the de novo generation of protease-resistant prion protein in yeast cells. *Biochemistry*, **45**, 6674-6680.

Treiber, C., Thompsett, A.R., Pipkorn, R., Brown, D.R. and Multhaup, G. (2007) Real-time kinetics of discontinuous and highly conformational metal-ion binding sites of prion protein. *J Biol Inorg Chem*, **12**, 711-720.

Tsenkova, R.N., Iordanova, I.K., Toyoda, K. and Brown, D.R. (2004) Prion protein fate governed by metal binding. *Biochem Biophys Res Commun*, **325**, 1005-1012.

Turnbull, S., Tabner, B.J., Brown, D.R. and Allsop, D. (2003a) Copper-dependent generation of hydrogen peroxide from the toxic prion protein fragment PrP106-126. *Neurosci Lett*, **336**, 159-162.

Turnbull, S., Tabner, B.J., Brown, D.R. and Allsop, D. (2003b) Generation of hydrogen peroxide from mutant forms of the prion protein fragment PrP121-231. *Biochemistry*, **42**, 7675-7681.

Turnbull, S., Tabner, B.J., Brown, D.R. and Allsop, D. (2003c) Quinacrine acts as an antioxidant and reduces the toxicity of the prion peptide PrP106-126. *Neuroreport*, **14**, 1743-1745.

Tuzi, N.L., Cancellotti, E., Baybutt, H., Blackford, L., Bradford, B., Plinston, C., Coghill, A., Hart, P., Piccardo, P., Barron, R.M. and Manson, J.C. (2008)

Host PrP glycosylation: a major factor determining the outcome of prion infection. *PLoS Biol*, **6**, e100.

Unterberger, U., Hoftberger, R., Gelpi, E., Flicker, H., Budka, H. and Voigtlander, T. (2006) Endoplasmic reticulum stress features are prominent in Alzheimer disease but not in prion diseases in vivo. *J Neuropathol Exp Neurol*, **65**, 348-357.

Uppington, K.M. and Brown, D.R. (2008) Resistance of cell lines to prion toxicity aided by phospho-ERK expression. *J Neurochem*.

Urano, S., Sato, Y., Otonari, T., Makabe, S., Suzuki, S., Ogata, M. and Endo, T. (1998) Aging and oxidative stress in neurodegeneration. *Biofactors*, **7**, 103-112.

Vanik, D.L., Surewicz, K.A. and Surewicz, W.K. (2004) Molecular basis of barriers for interspecies transmissibility of mammalian prions. *Mol Cell*, **14**, 139-145.

Varela-Nallar, L., Toledo, E.M., Larrondo, L.F., Cabral, A.L., Martins, V.R. and Inestrosa, N.C. (2006) Induction of cellular prion protein gene expression by copper in neurons. *Am J Physiol Cell Physiol*, **290**, C271-281.

Villa, V., Corsaro, A., Thellung, S., Paludi, D., Chiovitti, K., Venezia, V., Nizzari, M., Russo, C., Schettini, G., Aceto, A. and Florio, T. (2006) Characterization of the proapoptotic intracellular mechanisms induced by a toxic conformer of the recombinant human prion protein fragment 90-231. *Ann N Y Acad Sci*, **1090**, 276-291.

Voigtlander, T., Kloppel, S., Birner, P., Jarius, C., Flicker, H., Verghese-Nikolakaki, S., Sklaviadis, T., Guentchev, M. and Budka, H. (2001) Marked increase of neuronal prion protein immunoreactivity in Alzheimer's disease and human prion diseases. *Acta Neuropathol*, **101**, 417-423.

Walter, E.D., Chattopadhyay, M. and Millhauser, G.L. (2006) The affinity of copper binding to the prion protein octarepeat domain: evidence for negative cooperativity. *Biochemistry*, **45**, 13083-13092.

Watt, N.T., Routledge, M.N., Wild, C.P. and Hooper, N.M. (2007) Cellular prion protein protects against reactive-oxygen-species-induced DNA damage. *Free Radic Biol Med*, **43**, 959-967.

Watzlawik, J., Skora, L., Frense, D., Griesinger, C., Zweckstetter, M., Schulz-Schaeffer, W.J. and Kramer, M.L. (2006) Prion protein helix1 promotes aggregation but is not converted into beta-sheet. *J Biol Chem*, **281**, 30242-30250.

Webb, S., Lekishvili, T., Loeschner, C., Sellarajah, S., Prelli, F., Wisniewski, T., Gilbert, I.H. and Brown, D.R. (2007) Mechanistic insights into the cure of prion disease by novel antiprion compounds. *J Virol*, **81**, 10729-10741.

Weber, P., Giese, A., Piening, N., Mitteregger, G., Thomzig, A., Beekes, M. and Kretzschmar, H.A. (2007) Generation of genuine prion infectivity by serial PMCA. *Vet Microbiol*, **123**, 346-357.

Weise, J., Sandau, R., Schwarting, S., Crome, O., Wrede, A., Schulz-Schaeffer, W., Zerr, I. and Bahr, M. (2006) Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic caspase-3 activation, and exacerbation of ischemic brain injury. *Stroke*, **37**, 1296-1300.

Weissmann, C., Bueler, H., Fischer, M., Sailer, A., Aguzzi, A. and Aguet, M. (1994) PrP-deficient mice are resistant to scrapie. *Ann N Y Acad Sci*, **724**, 235-240.

Westaway, D., Cooper, C., Turner, S., Da Costa, M., Carlson, G.A. and Prusiner, S.B. (1994) Structure and polymorphism of the mouse prion protein gene. *Proc Natl Acad Sci U S A*, **91**, 6418-6422.

Westergard, L., Christensen, H.M. and Harris, D.A. (2007) The cellular prion protein (PrP(C)): its physiological function and role in disease. *Biochim Biophys Acta*, **1772**, 629-644.

White, A.R., Enever, P., Tayebi, M., Mushens, R., Linehan, J., Brandner, S., Anstee, D., Collinge, J. and Hawke, S. (2003) Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature*, **422**, 80-83.

White, A.R., Guirguis, R., Brazier, M.W., Jobling, M.F., Hill, A.F., Beyreuther, K., Barrow, C.J., Masters, C.L., Collins, S.J. and Cappai, R. (2001) Sublethal concentrations of prion peptide PrP106-126 or the amyloid beta peptide of Alzheimer's disease activates expression of proapoptotic markers in primary cortical neurons. *Neurobiol Dis*, **8**, 299-316.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P.G. (1996) A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*, **347**, 921-925.

Will, R.G. and Ward, H.J. (2004) Clinical features of variant Creutzfeldt-Jakob disease. *Curr Top Microbiol Immunol*, **284**, 121-132.

Wong, B.S., Brown, D.R., Pan, T., Whiteman, M., Liu, T., Bu, X., Li, R., Gambetti, P., Olesik, J., Rubenstein, R. and Sy, M.S. (2001a) Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. *J Neurochem*, **79**, 689-698.

Wong, B.S., Chen, S.G., Colucci, M., Xie, Z., Pan, T., Liu, T., Li, R., Gambetti, P., Sy, M.S. and Brown, D.R. (2001b) Aberrant metal binding by prion protein in human prion disease. *J Neurochem*, **78**, 1400-1408.

Wong, B.S., Liu, T., Li, R., Pan, T., Petersen, R.B., Smith, M.A., Gambetti, P., Perry, G., Manson, J.C., Brown, D.R. and Sy, M.S. (2001c) Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J Neurochem*, **76**, 565-572.

Wopfner, F., Weidenhofer, G., Schneider, R., von Brunn, A., Gilch, S., Schwarz, T.F., Werner, T. and Schatzl, H.M. (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J Mol Biol*, **289**, 1163-1178.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J. and Greenberg, M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326-1331.

Xing, H., Zhang, S., Weinheimer, C., Kovacs, A. and Muslin, A.J. (2000) 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. *Embo J*, **19**, 349-358.

Xiong, L.W., Raymond, L.D., Hayes, S.F., Raymond, G.J. and Caughey, B. (2001) Conformational change, aggregation and fibril formation induced by detergent treatments of cellular prion protein. *J Neurochem*, **79**, 669-678.

Yamaguchi, A., Tamatani, M., Matsuzaki, H., Namikawa, K., Kiyama, H., Vitek, M.P., Mitsuda, N. and Tohyama, M. (2001) Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. *J Biol Chem*, **276**, 5256-5264.

Yin, S., Pham, N., Yu, S., Li, C., Wong, P., Chang, B., Kang, S.C., Biasini, E., Tien, P., Harris, D.A. and Sy, M.S. (2007) Human prion proteins with pathogenic mutations share common conformational changes resulting in enhanced binding to glycosaminoglycans. *Proc Natl Acad Sci U S A*, **104**, 7546-7551.

Yu, S., Yin, S., Li, C., Wong, P., Chang, B., Xiao, F., Kang, S.C., Yan, H., Xiao, G., Tien, P. and Sy, M.S. (2007) Aggregation of prion protein with insertion mutations is proportional to the number of inserts. *Biochem J*, **403**, 343-351.

Yun, S.W., Gerlach, M., Riederer, P. and Klein, M.A. (2006) Oxidative stress in the brain at early preclinical stages of mouse scrapie. *Exp Neurol*, **201**, 90-98.

Zahn, R. (2003) The octapeptide repeats in mammalian prion protein constitute a pH-dependent folding and aggregation site. *J Mol Biol*, **334**, 477-488.

Zahn, R., Liu, A., Luhrs, T., Riek, R., von Schroetter, C., Lopez Garcia, F., Billeter, M., Calzolai, L., Wider, G. and Wuthrich, K. (2000) NMR solution structure of the human prion protein. *Proc Natl Acad Sci U S A*, **97**, 145-150.

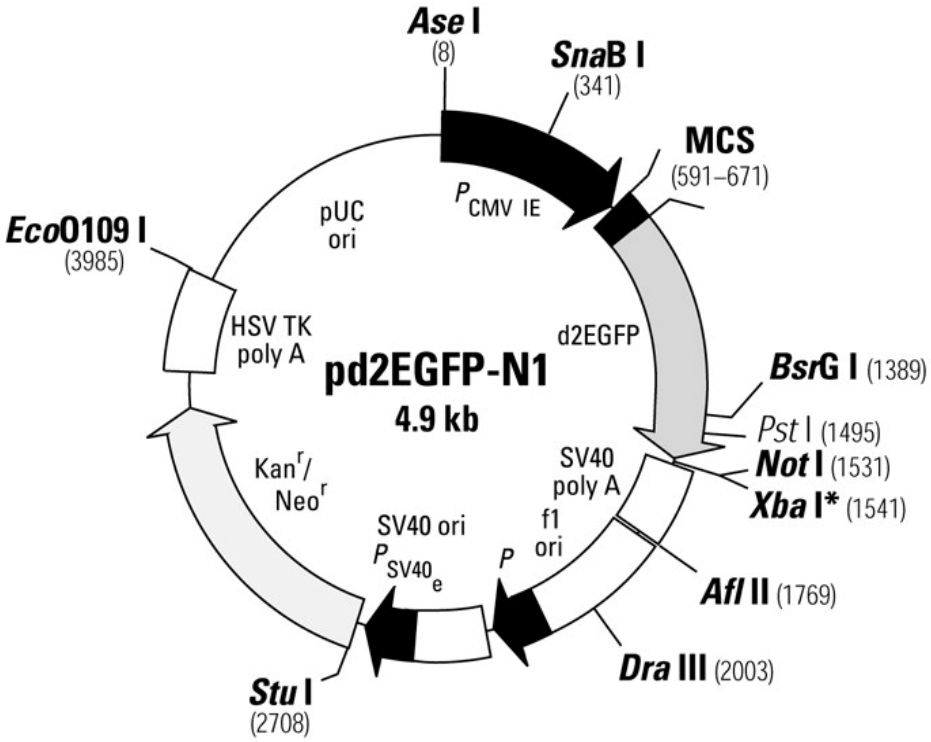
Zanuy, D., Gunasekaran, K., Ma, B., Tsai, H.H., Tsai, C.J. and Nussinov, R. (2004) Insights into amyloid structural formation and assembly through computational approaches. *Amyloid*, **11**, 143-161.

Zawlik, I., Witusik, M., Hulas-Bigoszewska, K., Piaskowski, S., Szybka, M., Golanska, E., Liberski, P.P. and Rieske, P. (2006) Regulation of PrPC expression: nerve growth factor (NGF) activates the prion gene promoter through the MEK1 pathway in PC12 cells. *Neurosci Lett*, **400**, 58-62.

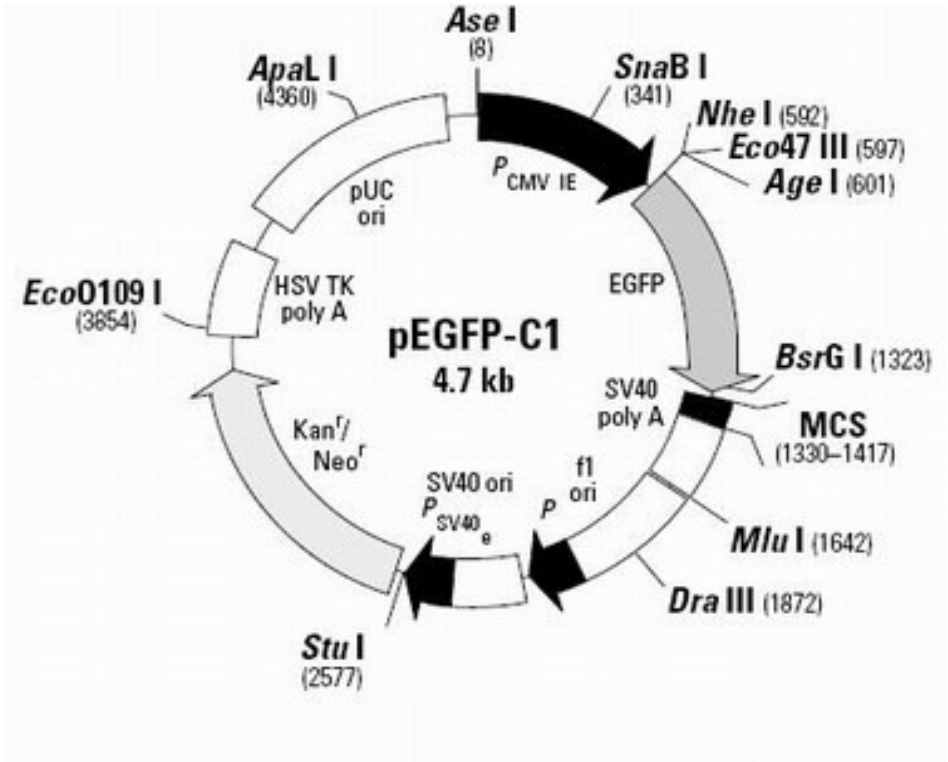
Zhu, F., Davies, P., Thompsett, A.R., Kelly, S.M., Tranter, G.E., Hecht, L., Isaacs, N.W., Brown, D.R. and Barron, L.D. (2008) Raman optical activity and circular dichroism reveal dramatic differences in the influence of divalent copper and manganese ions on prion protein folding. *Biochemistry*, **47**, 2510-2517.

Zou, W.Q., Capellari, S., Parchi, P., Sy, M.S., Gambetti, P. and Chen, S.G. (2003) Identification of novel proteinase K-resistant C-terminal fragments of PrP in Creutzfeldt-Jakob disease. *J Biol Chem*, **278**, 40429-40436.

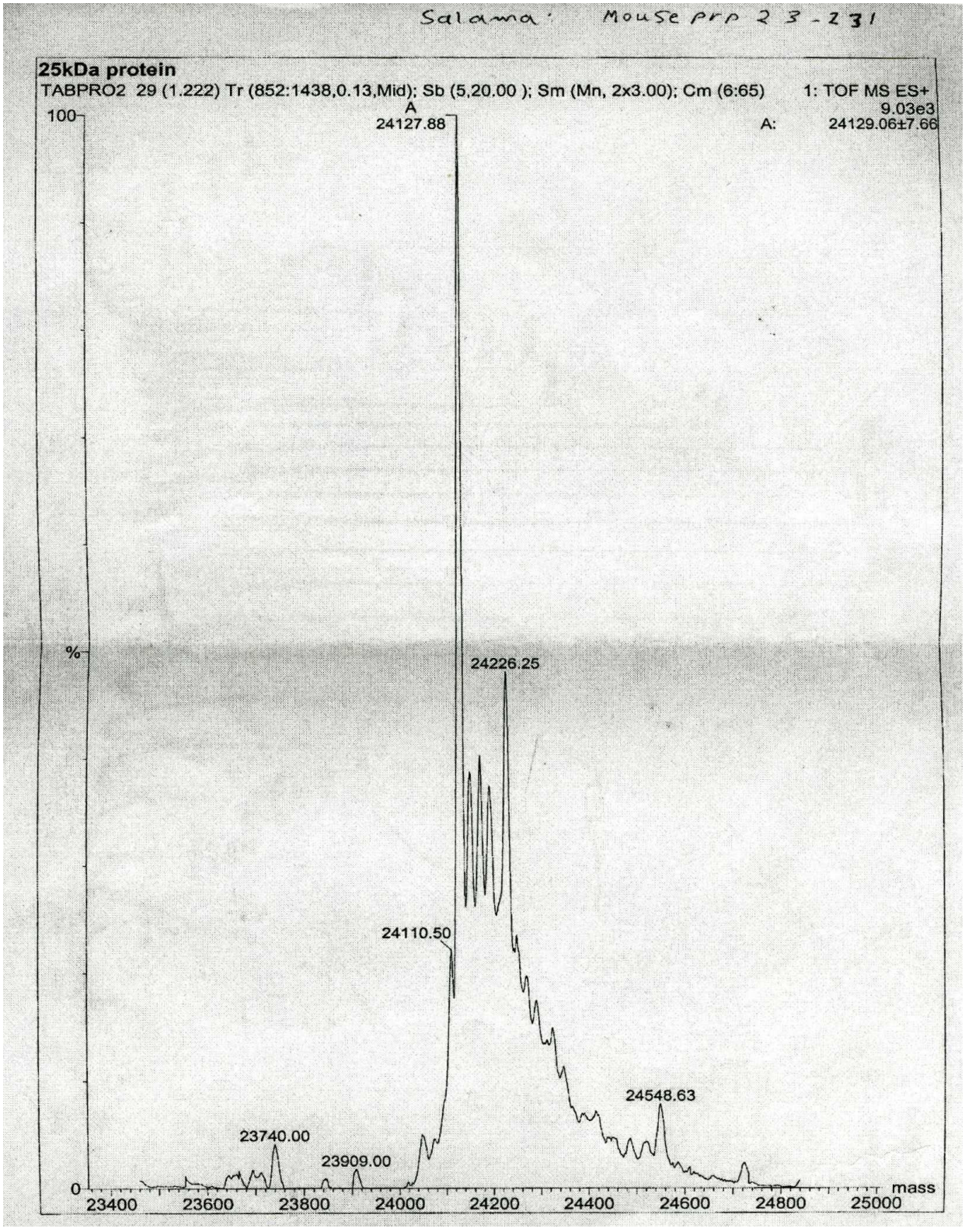
Appendices



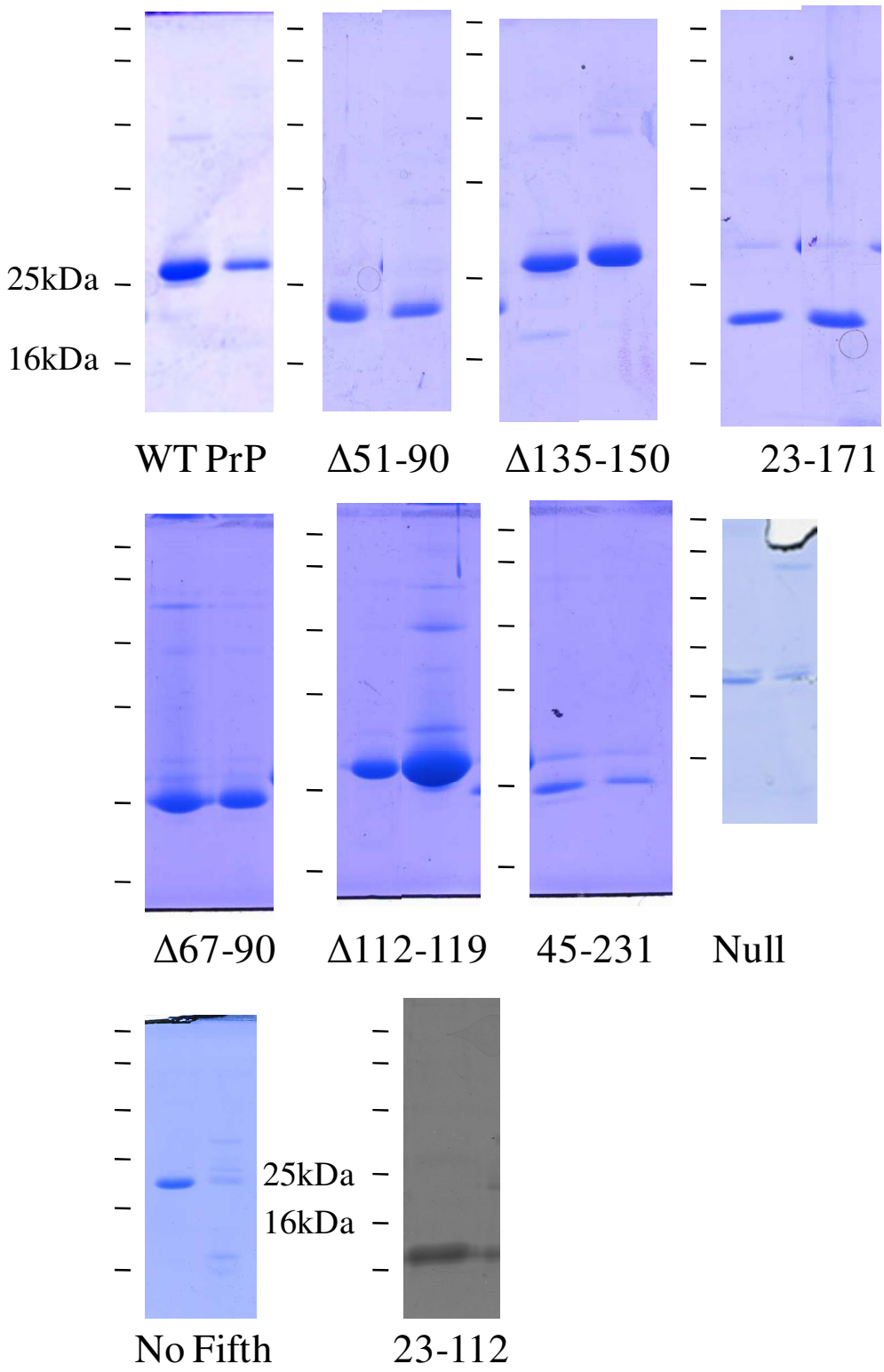
Schematic diagram of the pd2-EGFP-N1 plasmid used for the promoter constructs.



Schematic diagram of the pd2-EGFP-N1 plasmid used for the mouse, Xenopus and turtle PrP constructs.



Mass spectroscopy of full-length his-tagged mouse PrP.



Gels of manganese bound proteins, first band shows manganese bound protein second band shows metal free protein. Bottom marker represents 16kDa, excluding 23-112.